Fine Mapping two resistance QTLs to *Puccina graminis* f.sp. *avenae* and evaluating adult susceptibility in Barley

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20.07.2016

Abstract

In the past, resistance quantitative trait loci (QTL) were mapped in barley (*Hordeum vulgare L.*) against several heterologous rust fungi. Recently, super susceptible barley line SusPtrit was tested for susceptibility to heterologous pathogen *Puccinia graminis* f.sp. *avenae (Pga)* using isolates Ingeberga, Pattala and Evertsholm. The results showed that SusPrit was resistant against *Pga* Evertesholm and susceptible to *Pga* Ingeberga and *Pga* Pattala. This was the first account that SusPtrit was resistant to one isolate and susceptible to all other tested isolates of the same pathogen. Interval mapping of the segregating barley population Vada x SusPtrit (VxS) mapped ten QTLs *(Rpgaq1-Rpgaq10)* effective against tested *Pga* isolates. The presented study fine maps the only two QTLs (*Rpgaq1* and *Rpgaq5*) that showed effectiveness against *Pga* Evertsholm. Both QTLs possess a dominant gene action and the positions of *Rpgaq1* and *Rpgaq5* were narrowed down to 1 cM and 2 cM respectively. It remains unclear if minor QTLs are segregating in the mapping population. Also, the low infection levels of *Pga* Evertsholm might have affected the prediction of QTLs in the VxS mapping population. Generally, it remains questionable if the number of individuals needed to map QTLs was statistically sound, because the number needed increases as the average phenotype difference between genotype groups decreases.

Further, we confirmed previous observations of adult plant susceptibility to *Pga* on volunteer barley plants, using five near non-host barley accessions inoculated at different developmental stages. So far, only seedlings were studied and confirmed to be infected by heterologous rusts. The presented adult plant test showed that barley accession L94 was susceptible to infection with *Pga* Ingerberga at the adult plant stage. Unfortunately, a contamination with an unknown disease, against which L94 seemed resistant, did not allow the observation of adult susceptibility in the four additional barley accessions. However, based on the results of L94 we observed that the infection was evident but lower than the control oat plants. Also, the stem infection of L94 already occurred at earlier developmental stages than control oat plants, however the infection of L94 was no longer present in the latest developmental stages. We concluded that the infection with *Pga* is possible in the adult plant stage, however the infections are most pronounced during the seedling stage. It was not possible to conclude on the adult plant susceptibility relative between the different barley accessions.

Inhaltsverzeichnis

1. Introduction

1.1. *Puccinia spp.* and durable Resistance

From an agricultural perspective, rust pathogens (*Puccinia* ssp.), are a major threat to various crops globally, including wheat, barley, and oat, due to their debilitating effect on yield. Among others, sexual reproduction and extreme mobility of rust spores facilitate gene flow, and the spread of loss of function mutations in *Avr* genes (Morjan et al. 2004). Often, new and constantly evolving pathogen genotypes break monogenic resistances in crop cultivars (Morjan et al. 2004). This suggests that resistance governed by the gene-for-gene relationship is vulnerable to break down due to rapidly evolving rust pathogens. Therefore, more durable forms of resistance are required to protect cereal crops.

In theory, the most durable resistance is non-host resistance (NHR), which is defined as a situation where all genotypes of a plant species are immune to all genotypes of a pathogen species (Nuernberger and Lipka 2005; Heath 2002, Niks et al. 2014). However, elucidating the genetic basis of NHR against heterologous pathogens requires inter-species crosses, which are notoriously difficult to achieve (Acquaah, 2012). But, similarities to NHR are observed in near non-host resistance (NNHR), which is defined as a situation where most genotypes of a plant species are immune to a heterologous pathogens, and only a few genotypes show moderate to low infection (Niks 1987). Histological studies showed that both NHR and NNHR share resistance mechanisms on a cellular basis, proposing similarities in the pathogen suppression (Heath 2000). Further, contrary to NHR, NNHR allows intra-species crosses, which are easier to perform, compared to inter-species crosses (Acquaah, 2012). Therefore, inheritance studies are performed using heterologous rust species of near non-hosts plant species to elucidate the resistance reaction and determine why pathogens supress basal defences in host plants, but fail to infect non-host plants.

1.2. Basal Resistance and Partial Resistance

In general, host resistance is observed as either a pre-haustorial resistance (considered a form of basal resistance) or as a post-haustorial hypersensitive response (Jafary et al. 2006). Moreover, successful infection requires pathogens to circumvent the basal defence to establish basic compatibility by overcoming physical and chemical constitutive barriers and induced defences, (Niks 2014). Histological studies showed that heterologous rusts penetrate the leaves of immune and resistant near non host species at the same frequency as host species (Dracatos et al. 2016). Therefore, the pathogen is not merely suppressed due to an inability to penetrate the leaf. Rather, a common basal defence against heterologous and homologous rusts alike is expressed through failed haustorium formation, connected to cell wall reinforcements called papillae (Niks 2014). This is a pre-haustorial response and occurs mostly without the induction of a hypersensitive response (HR) (post-haustorial) (Mellersh and Heath 2001, Niks 1983). However, in case of occasional haustorial formation, post-haustorial HR may act as a secondary defence. (Christopher-Kozjan and Heath, 2003). Overall, Haustorium formation is almost entirely prevented by NHR but only hampered to some extent against adapted pathogen attacks, resulting in partial resistance (PR) (Niks 1982; Hoogkamp et al. 1998). Molecular genetic studies report that several QTLs mapped in relation to PR also showed effectiveness against heterologous rust, this suggests a genetic association of PR and NHR at the level of basal resistance (Jafary et al. 2006, Marcel et al. 2006).

1.3. Specificity of Non-Host Resistance in the Barley-Rust Pathosystem

In barley, 109 accessions were screened with the heterologous wheat leaf rust *Puccinia triticina (Pt)* which identified several accessions with moderate to low susceptibility (Atienza et al. 2004). The four most susceptible accessions were double crossed and subjected to pedigree selection, which resulted in the experimental line SusPtrit. Accumulated susceptibility alleles render it just as susceptible as host species in the seedling stage (Atienza et al. 2004). Additionally, some accessions that were susceptible to one heterologous rust were often also susceptible to other heterologous rusts (Atienza et al. 2004)

Consequently, an intraspecific cross between susceptible SusPtrit and immune Vada (VxS) barley accessions allowed the development of a mapping population to observe the inheritance of NNHR (Jafary et al. 2006). Consequently, 152 VxS recombinant inbred lines (RILs) led to the mapping of 18 chromosomal regions active against 10 homologous and heterologous rust species (Jafary et al. 2006). Eleven chromosomal regions were effective to only one rust species while seven demonstrated effectiveness against more than one rust species (Jafary et al. 2006). It was concluded that NNHR is controlled by QTLs with different and overlapping specificities. Overall, NHR is regulated by multiple QTLs plus occasional R-genes (Niks 2014). Following the work on the VxS mapping population, Jafary et al. (2008) developed an additional mapping population using immune Cebada Capa x SusPtrit to map resistance QTLs. However, QTLs for resistance in Vada are mostly different from those in Cebada Capa (Jafary et al. 2008). Thus, there is an abundance of loci carrying alleles for NNHR, very similar to the abundance of loci in PR (Jafary et al. 2008; Marcel et al. 200&). One explanation is that the QTLs accumulated effect is difficult to supress because the pathogen would have to render each individual resistance-allele ineffective (Jafary et al. 2008).

1.4. Mapping Resistance in Barley to Oat Stem Rust

Currently, efforts are still underway to clone large effect genes that underpin QTLs for resistance against the heterologous wheat leaf rust pathogen *Puccinia triticina* in barley. However, additional rust species have also been included in the general investigation of non-host resistance and the presented thesis focuses on the oat stem rust pathogen *Puccinia graminis* f. sp. Avenae *(Pga).* One of the first documentations of *Pga* infections of heterologous plant species was noticed by Martens et al. (1977) on volunteer barley plants. Surprisingly, Martens et al. (1977) seemed to have found an adult plant in the field that was moderately susceptible to *Pga*. In our experience, the susceptibility of SusPtrit to heterologous rusts pertains to the seedling stage (Atienza et al. 2004). In any case, identifying resistance QTLs for oat stem rust in barley could lead to finding homologs in oat, which could provide measures against the erosion of monogenic resistances witnessed in Australia (Dracatos personal communication)

Recently, Dracatos et al. (2014) found five minor effect genes effective against all tested *Pga* isolates in a Yerong x Franklin double haploid population. A histological analysis demonstrated that the resistance was pre-haustorial in response to *Pga.* Additionally, Dracatos et al. (2016) tested *Pga* on a subset of the 109 accessions tested by Atienza et al (2004), and 11% of the subset was susceptible, concluding that barley is a near non-host of *Pga*. SusPtrit was tested with three single pustule *Pga* isolates and was susceptible to *Pga* isolates Pattala and Ingeberga but resistant to *Pga* isolate Evertsholm (Dracatos et al. 2016). The resistance of SusPtrit to *Pga* Evertsholm is remarkable because it is the first time that SusPtrit was fully resistant to one isolate of a heterologous pathogen, and susceptible to all other tested isolates of that same pathogen.

The three isolates, were studied in three mapping populations (Vada x SusPtrit, Cebada Capa x SusPtrit, and SusPtrit x Golden Promise) to identify resistance genes and dissect the species specificity (Dracatos et al. 2016). It was concluded that as a near non-host, barley is affected by *Pga* both in a specific and non-specific manner and that only a few QTLs overlapped between populations (Dracatos et al. 2016). Interval mapping identified ten QTLs *(Rpgaq1-Rpgaq10*), effective against the tested *Pga* isolates. Of the ten QTLs, five were mapped in the Vada x SusPtrit (VxS) mapping population (Dracatos et al. 2016). Specifically, *Rpgaq1* and *Rpgaq5* were identified as large effect QTLs in the VxS cross, effecting *Pga* Evertsholm with significant LOD peaks suitable for fine mapping (Figure 1). Dracatos et al (2016) reports that *Rpgaq1* is donated by immune Vada and is isolate non-specific, while *Rpgaq5* is donated by SusPtrit and is isolate specific with signs of quantitative isolate specificity. Available fine mapping crosses for each QTL were selected to narrow down the location of both large effect QTLs. Overall, using the VxS mapping population and the *Pga* isolate Evertsholm allows resource efficient fine mapping of both QTLs.

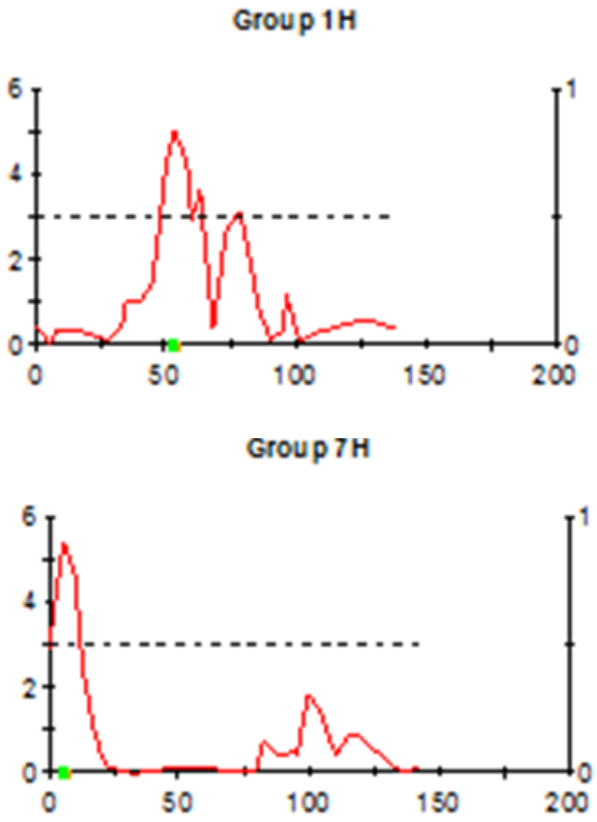


Figure 1: LOD profiles reported by Dracatos et al. 2016 for *Rpgaq1* on chromosome 1H and *Rpgaq5* on chromosome 7H.

1.5. Objectives of Presented Thesis

The main objective of the presented study is to narrow down the interval size on the linkage map for the proposed QTLs, to a point where physical mapping is possible so available BAC libraries for Vada and SusPtrit can be used. Eventually this should enable the map based cloning of both candidate genes. The material used by Dracatos et al. 2016 was used to ensure a seamless transition from the QTL analysis to the fine mapping of identified QTLs.

Additionally an adult plant test will clarify if barley accessions are susceptible to *Pga* at both the seedling stage and the adult plant stage. Because Martens et al. (1977) found naturally infected barley volunteer plants with *Pga* in the field, the question remains if adult plants can also be infected. Previously it was concluded that partial resistance genes act in a developmental stage dependent manner after infection with *P. hordei* (Wang et al. 2010). To test the susceptibility of the plants at various developmental stages we will inoculate various developmental stages with *Pga* isolate Ingeberga. According to Dracatos et al. (unpublished), *Pga* Ingeberga was considerably more virulent than *Pga* Evertsholm. All studied barley accessions in the presented adult plant test were most susceptible to *Pga* Ingeberga at the seedling stage (Dracatos et al. unpublished). Thus, we want to observe if susceptible seedlings also lead to susceptible adult plants. This will help to study nonhost resistance at the adult plant stage.

2. Materials and Methods

2.1. Fine Mapping Methodology

2.1.1. Plant material

Three interesting RILs were selected by Nansamba (unpublished) to make crosses between susceptible and resistant RILs (Table 1). RIL 152 did not contain a resistance allele for either QTL, and was therefore used as a parent in both crosses. RIL 110 carries a resistance allele for *Rpgaq1*, and RIL 143 carries a resistance allele for *Rpgaq5* (Table 1). Using RIL 152 as a parent in both crosses ensured segregation for only one QTL. Each cross created the basis for a mapping population of each studied QTL (*Rpgaq1* and *Rpgaq5*). Cebeco Oat and RIL 152 were used as susceptible controls to confirm successful infection with *Pga* Evertshom at a reasonable density. While immune barley variety Vada, and RIL 110 and RIL 143, were used as resistant controls to confirm there was no contamination with *Puccinia hordei*. RIL 110 and RIL 143 were used as resistant controls in the *Rpgaq1* and *Rpgaq5* mapping population respectively. Unlike Cebeco Oat and Vada, RIL 152, RIL 110 and RIL 143 were only used as controls in the 3rd seedling test. Ten days after sowing the seedlings were inoculated on the first leaf.

	RIL Cross	Linkage Group/QTL	Genotype	Resistance allele donor
	152	1H/*Rpgaq1*	$r_1r_1r_5r_5$	Vada
x	110		$R_1R_1r_5r_5$	
	143	7H/*Rpgaq5*	$r_1r_1R_5R_5$	SusPtrit
x	152		$r_1r_1r_5r_5$	

Table 1: fine mapping crosses for *Rpgaq1* and *Rpgaq5*

2.1.2. Oat Stem Rust Pathogen

The studied *Puccinia graminis* f. sp. *avenae* isolate was collected as a field isolate at Evertsholm, Sweden, and at Wageningen it was multiplied on Alfred oat, and here a single pustule isolate was drawn from oat. This single pustule isolate was not virulent on SusPtrit. Disease tests were performed on segregating recombinants (3.3), the first seedling test (3.4), the second seedling test (3.5) and the third seedling test (3.7). However, we have slightly different spore origins to report. For the recombinant screening (3.3), the first seedling test (3.4), and the third seedling test (3.7), *Pga* isolate Evertsholm was maintained on Cebeco oat, in a greenhouse compartment and periodically collected using a cyclone spore collector. Then harvested spores were weighed, and stored up to 14 days in a desiccator at room temperature. Access spores were transferred to liquid nitrogen and stored in a -80°C freezer. The second seedling test (3.5) however, relied on 5 ampules (10 to 20 mg) from different multiplication trials stored at -80°C. The spores were thawed quickly using hot water to ensure high viability. It should be noted, that it is a lengthy process for sufficient fresh stem rust to develop on the host (~21days).

1.1.1. Inoculation

In the inoculation tray, the first leaves of each seedling were pinned horizontally to the soil, using small metal loops with the adaxial side facing up. All later developed leaves were removed. For the first seedling test (3.4) and segregating recombinants (3.3), and the third seedling test (3.7) every box was inoculated with 8mg of fresh uredospore mixed with ~92 mg of lycopodium, and applied using a settling tower. This process facilitated a uniform distribution of approximately 460 uredospore per cm^2. For the second seedling test (3.5) we distributed 10 mg of previously frozen uredospore diluted with ~90 mg lycopodium per tray. For all inoculations, germination took place at 100% relative

humidity, complete darkness, and temperatures of 17-18°C in a humidity chamber overnight. Microscope slides were place on each tray before inoculation in the settling tower and evaluated after the overnight germination period in the humidity chamber. Next, the slides were evaluated using a microscope, and checked for successful hyphae formation by the rust spores.

1.1.2. DNA Isolation and Genotyping

Approximately 10 days after inoculation, we conducted the DNA isolation. For this, two cm^2 of the second leaf was removed from each plant and stored in a cluster tube. The cluster tubes were kept on ice and each contained two stainless steel balls (2mm) and the isolation buffer explained by Wang (personal communication). After all plants were sampled, the cluster tubes were placed in a lyzermachine. The cluster tubes containing the lyzed cells were then centrifuged at 2000 rpms and next placed in a 100 °C water bath for 5 minutes. Afterwards, the cluster tubes were centrifuged again at 4040 rpm for 5-10 minutes. Finally, the supernatant in each tube was collected and transferred onto a 96 well PCR plate and diluted 4 fold. For best results, a second 96 well PCR plate was prepared where the isolated DNA was diluted another 10 fold (Wang personal communication).

Genotyping was done using the Lightsanner system© which uses a fluorescent double-strand DNA (dsDNA) binding dye (LCGreen) and a PCR product. The dsDNA binding dye enables Hi-Res Melting curve analysis and detects DNA sequence variants. LCGreen is unique in its ability to detect the presence of heteroduplexes formed during PCR. Images of DNA melting are captured by a charged coupled device camera (CCD), and magnified to reveal DNA melting profiles. Sample-to-sample comparisons of these images were then used to interpret allele composition of the amplified DNA fragments. Using heterozygous parent DNA it was possible to distinguish between each homozygous parent as well as heteroduplexes representing heterozygotes. From these comparisons, proposed markers will be confirmed or rejected for being polymorphic between the parents, and confirm SNPs used to genotype tested individuals

1.1.3.Phenotyping

Quantifying the level of infection was done twelve days after infection (dai). The progeny of segregating recombinants from recombinant screening (3.3) and the first seedling test (3.4) were phenotyped only for the presence or absence of pustules. The number of pustules per cm^2 at the top, middle and bottom of the leaf were recorded and averaged. Because the flecks were not included in the phenotyping, the VIS (visual infection sites) could not be recorded because it is the sum of flecks (>5mm) and pustules. In any case, we described affected individuals as being resistant (R) or susceptible (S) based on the presence of pustules or not. Though, due to the missing observations on the occurrence of flecks (>0.5mm), R is not reliable and might encompass an individual that exhibited flecks, actually rendering it S. Thus only S is a reliable phenotype and R will always require uncertain judgment to confirm result and support the fine mapping of both QTLs. The second seedling test (3.5) phenotyped the tested individuals on the basis of VIS, observing both pustules and flecks (>0.5mm). Each leaf was assessed whole for VIS and the length of the leaf was recorded. This made it possible to describe the VIS according to the length of the leaf (VIS/cm leaf). Finally, the third seedling test (3.7) was observed in the same manner as the second seedling test, but complemented with the measurement of the leaf width (measured at the widest point), this allowed the calculation of the VIS/cm^2. For those families obviously demonstrating full susceptibility, only 15 plants were phenotyped for VIS/cm^2.

1.1.4.Marker Development and SNP distance calculation

In all cases, markers were based on single nucleotide polymorphism (SNP). Initially, primers developed by Nansamba (unpublished) for SNPs in VxS SNP map were tested. Later new primers were developed for SNPs located in the target region of the VxS SNP map. This was done according to the short protocol: first SNPs are selected for the target region from the VxS SNP map and then blasted against Morex, Barke and Bowman sequence to ensure marker specificity to the targeted locus. Next Primers with 20-30 bases and a melting temperatures of 58-60 °C were developed using Primer3Plus©. The primers were tested using DNA from Vada and SusPtrit while the PCR was run at a higher hybridization temperature (68°C) if non-polymorphism was reported at 60°C hybridization temperature. Only polymorphic markers were selected (see 3.1). Marker distances were calculated according to the recombination frequencies between SNP following genotyping.

2.1.3.Recombinant Screening

For the recombinant screening, fully heterozygous F_2 individuals for the target region of *Rpgaq1* and *Rpgaq5* respectively were selfed. Each family was grown in trays with 77 single, individually labelled pots. Ten days after sowing, plants were genotyped to identify heterozygous recombinants. They were placed in two groups: 1) segregating recombinants: heterozygous recombinants with homozygous susceptible stretches and 2) resistant recombinants: heterozygous recombinants with homozygous resistant stretches. The resistant heterozygous recombinants, were directly transplanted in pots to set seed, because a QTL will contribute a resistance allele regardless if it maps to homozygous or heterozygous stretches, therefore only showing resistant phenotypes, hindering the localisation of the QTL. All segregating heterozygous recombinants on the other hand were inoculated with *Pga* Evertsholm and phenotyped 10 days after inoculation. Segregating recombinants will be susceptible or resistant depending if the QTL is located in the homozygous stretch lacking a resistance allele, or located in the heterozygous stretch carrying a resistance allele. On a single plant basis, the phenotype and recombination position of potentially susceptible heterozygous recombinants were compared to infer QTL position.

Cross/Mapping Population	**Segregating recombinants**	**Resistant recombinant**
152 x 110 / *Rpgaq1*	SusPtrit-Heterozygous (sh)	Vada-Heterozygous (vh)
143 x 152 / *Rpgaq5*	Vada-Heterozygous (vh)	SusPtrit-Heterozygous (sh)

Table 2: Genetic susceptibility of Recombinants

For *Rpgaq1*, Vada is the resistance donor demonstrating a dominant gene action. Hence, all Vada-heterozygous recombinants (vh recombinants) are expected to be fully resistant because an *Rpgaq1* resistance allele is expressed by heterozygous and homozygous stretches alike (Table 2). However if vh recombinants are selfed, the homozygous recombinants can be used to infer QTL position, depending if the QTL maps to the homozygous resistant or homozygous susceptible stretch. For SusPtrit-heterozygous recombinants (sh recombinants) on the other hand, the genotype of the QTL location conditions segregation into susceptible or resistant phenotypes (Table 2). We inoculated sh-recombinants with *Pga* Evertsholm to infer the QTL position. Susceptible sh-recombinants are homozygous SusPtrit for *Rpgaq1* because they are missing the resistance allele, while the *Rpgaq1* locus maps to the heterozygous stretch if the phenotype is resistant and therefore carrying a resistance allele. Comparing the phenotype and genotype of individual sh recombinants sufficed to map *Rpgaq1*, and no selfing was needed. Hence, using sh recombinants with recombinantions in different marker intervals allows the mapping of *Rpgaq1* following inoculation. However, mapping based on single plants is less reliable than based on entire families.

For *Rpgaq5*, SusPtrit is the resistance donor, which also demonstrated a dominant gene action. However, reciprocal to *Rpgaq1*, the sh recombinants were resistant; heterozygous and homozygous stretches can not be differentiated by the phenotype (Table 2). However, the inoculation of vh recombinants results in segregating phenotypes, which are therefore segregating recombinants (Table 2). The susceptible vh recombinants are homozygous Vada for the QTL location while the resistant vh recombinants are heterozygous for the QTL location. The principle is the same as explained above. For later seedling tests, all identified recombinants were archived directly or after inoculation.

1.1.5. Seedling Test

The seedling tests are essentially disease tests conducted in inoculation trays containing 30-40 seedlings. For each seedling test the progeny of heterozygous recombinants were sown as families into inoculation trays. Heterozygous recombinants with the recombination in different marker intervals were used to infer the QTL position. Fully susceptible or fully resistant families contain the QTL in the homozygous stretch while segregating families suggest the QTL location in the heterozygous stretch. According to a Student's T-test ($p<0.05$), fully susceptible and fully resistant families do not have significant differences between average means of the genotype groups, while segregating families show a significant difference between the average means of the homozygous Vada and homozygous SusPtrit genotype group. Cebeco oat was used as a susceptible control to confirm successful inoculation with *Pga* isolate Evertsholm at a sufficient and appropriate density. Vada was used as a resistant/immune control. An infection of Vada would have suggested cross contamination by *Puccinia hordei* frequently used in Wageningen Unifarm. The trays are then inoculated and later phenotyped. In the third seedling test RIL 152, 110 and 143 complemented Cebeco Oat and Vada for additional control as described above. We strongly recommend to use these additional controls and only fresh spores in the future.

1.2. Adult Plant Test

1.2.1. Plant Material

Five barley accessions (Trigo Biasa, SusPtrit, SusPmur, VxS RIL 152 and L94), one Wheat accession (8860) and 3 control oat accessions (Alfred, Swan and Cebeco) were sown at five seeds per pot and accession/sowing date. This was repeated in intervals of approximately 14 days. Overall, 6 sowing dates were inoculated with 2-5 plants per pot: 10 d, 24d, 37d, 51d, 65d and 79 d old individuals. All barley accessions were known to be susceptible to some degree to the heterologous rust *Puccinia graminis* f. sp. *avenae* at the seedling stage. Unfortunately, not all plants germinated as expected. Swan Oat germinated poorly and was later omitted from the results because most sowing dates showed little or no germination.

1.2.2. Oat Stem Rust Pathogen

Puccinia graminis f. sp. a*venae* isolate Ingeberga was chosen for the adult plant test because it was more virulent than the other *Pga* isolates tested by Nansamba et al. (unpublished). The multiplication was done on Cebeco oat. Once sufficient sporulation was achieved the spores were harvested using a cyclone spore collector (~500mg). After collecting the spores, they were stored in a desiccator at room temperature and access spores were transferred to liquid nitrogen and stored in a -80°C freezer.

1.2.3. Inoculation and Observation

The inoculation was performed in a location without draft and inside a large box without a lid to ensure very little air movement and homogenous dissemination of the inoculum. The inoculation was performed per sowing date group. Due to the obvious size differences between early and late sowing dates of the populations, we used a progressive dose of inoculum: 10d = 30 mg spores + 300 mg lycopodium; 24d= 60 mg + 600 mg lycopodium and 37d, 51d, 65d, and 79d = 80 mg +800 mg lycopodium. As a group, the plants were sprayed with the inoculum by hand, using a handheld Kabierske powder blower. Using many angles, the inoculum was applied evenly onto all plants. Next, they were placed into a humidity chamber overnight at 100% relative humidity and temperatures of 17-18°C. Microscope slides were place in each pot before inoculation in the settling tower and evaluated after the overnight germination period. After germination was confirmed the plants were placed into a greenhouse compartment and covered with a frame wrapped in transparent foil, to keep an environment of prolonged high humidity especially important for the germination of stem rusts (Rowell, 1984). The frame was removed in the afternoon following the overnight germination.

Twelve days after inoculation, all plants were phenotyped. We recorded the approximate leaf infection (0, +5, +10, +15, +20, +30 pustules etc.), namely pustules and teliospores of all plants per pot and averaged the infection for each leaf stage. This information was complemented with pictures of the youngest leaves at the point of inoculation (i.e. 10d, 24d, 37d etc.). Furthermore, all plants were evaluated for their stem infection based on the frequency of the infection between all plants in one pot (% of plants showing stem infection). Furthermore, all infected stems were documented photographically to give a better impression of the infection amount on stems. Finally, comments were included about irregularities and striking observations. Only visual infection was observed, no histological studies were conducted

2. Results

2.1. SNP Test and F_2 Genotyping

All specific primers and the corresponding SNPs were evaluated for polymorphism between the parents. The polymorphic SNPs were used to improve the available genotyping of the F_2 generation in the *Rpgaq1* and *Rpgaq5* mapping populations (Table 3 and Table 4). The new information allowed the calculation of a preliminary linkage map (Figure 2a and 3a).

2.1.1. Fine mapping *Rpgaq1* based on F_2 population

In the *Rpgaq1* target region, Nansamba (unpublished) identified 13 SNPs using the VxS SNP map (Table 3). Of those, four SNPs were previously used to genotype the F_2 Generation (Nansamba unpublished) (Table 3). The remaining 9 SNPs were identified by Nansamba (unpublished) but the F_2 genotyping is attributed to the presented work (Table 3, Appendix 1A).

First, three markers were deemed unfit for genotyping due to non-polymorphism between the parents, either by the primer test reported by Nansamba et al. (unpublished) or by the presented confirmation of the primer test using two annealing temperatures (60 °C to 68 °C) (Table 3). The original left flanking marker (M1) showed that it was non-polymorphic at both tested temperatures and did not confirm results by Nansamba (unpublished) (Table 3). Nevertheless, we relied on the data provided for M1 but abandoned its use in future experiments (Niks personal communication). Additionally, as stated by Nansamba (unpublished), M3 and M5, were confirmed to be non-polymorphic (Table 3). Furthermore, M2 and M6 were only polymorphic between the parents if the primer annealing temperature was increased from 60 °C to 68 °C (Table 3). Therefore, we continued their application using 68 °C.

Marker	SNP ID	VxS SNP map (cM)	Nansamba Primer Test (at 60 C)	Confirmation	Chi Square test
M1	BOPA2_12_31276*	49.2	Polymorphic	non-polymorphic	0.557
M2	**SCRI_RS_116548**	**54.4**	**Polymorphic**	**polymorphic at 68 °C**	**0.481**
M3	SCRI_RS_232660	N/A	Non-polymorphic	confirmed	-
M4	**BOPA2_12_31177***	**55.5**	**Polymorphic**	**confirmed**	**0.388**
M5	SCRI_RS_193392	63.4	Non-polymorphic	confirmed	-
M6	**BOPA1_409-1643***	**70.7**	**Polymorphic**	**polymorphic at 68 °C**	**0.119**
M7	**BOPA2_12_30562**	**72.5**	**Polymorphic**	**confirmed**	**0.136**
M8	**BOPA2_12_10198**	**88.1**	**Polymorphic**	**confirmed**	**0.427**
M9	**SCRI_RS_156506**	**88.7**	**Polymorphic**	**confirmed**	**0.125**
M10	BOPA1_5768-469	95.9	Polymorphic	confirmed	0.002***
M11	**SCRI_RS_204611**	**99.7**	**Polymorphic**	**confirmed**	**0.132**
M12	BOPA1_12492-541*	109.3	Polymorphic	confirmed	0.011**
M13	SCRI_RS_139690	117.9	Polymorphic	confirmed	0.000***

Table 3: SNPs tested on parents and mapped in *Rpgaq1* mapping population to confirm polymorphism previously tested by Nansamba (unpublished). Bold rows were used to genotype F_2, non-bolded rows contain abandoned SNPs. *SNP used by Nansamba (unpublished) to genotype F_2. 1:2:1 segregation tested against Chi-square test ** tendency for significant difference ($p<0.01$), *** significantly different ($p<0.01$). All SNPs were tested at 60 °C

In the next step all polymorphic SNPs were genotyped, to complement F_2 genotyping results determined by Nansamba (unpublished) (Appendix 1). Using the Chi-Square test to statistically confirm a 1:2:1 allele segregation between the genotype groups (homozygous Vada, heterozygous, and homozygous SusPtrit), we noticed distorted segregation ($p<0.05$) favoring Vada alleles for M10 and M13, therefore they were excluded from further use (Table 3). Also, M12 was excluded because the Chi-square value ($p=0.011$) showed a tendency for distorted segregation also favoring Vada alleles (Table 3). The overrepresentation of Vada could be conditioned by genotyping error because differentiation between Vada and SusPtrit Lightscanner curves is more difficult than identifying the heteroduplex of heterozygous individuals. Also the ease of differentiating between parental Lightscanner curves differs between SNPs in general. In the end, we retained a set of five reliable SNPs consisting of M2, M7, M8, M9 and M11. This set was complemented with reliable genotyping data previously collected for M1, M4, M6 (Nansamba unpublished), to produce the overall F_2 genotyping (Appendix 1).

The linkage map construction produced a 49 cM target region based on the information from 70 plants (Figure 2A). Next, the VIS previously recorded by Nansamba (unpublished) was averaged per genotype group (v, h, and s) of each SNP and the phenotypic differences between the genotype groups were compared (Figure 2B). We found that all homozygous Vada groups were considerably less susceptible to *Pga* Evertsholm than homozygous SusPtrit genotype group, which was expected considering that Vada is the resistance donor for *Rpgaq1* (Figure 2B). Furthermore, across all SNPs in the *Rpgaq1* mapping population, M4 showed the lowest average VIS for the homozygous Vada genotype group, and one of the highest average VIS scores for the homozygous SusPtrit genotype group (Figure 2B). With increasing distance away from M4, the average VIS increased for homozygous Vada genotype groups per SNP (Figure 2B). Therefore, we propose that *Rpgaq1* is located in the interval between the flanking markers of M4, reducing the 49 cM interval to a 7 cM interval between M2 and M6 (Figure 2A). The average VIS for the heterozygous genotype group of M2, M4 and M6 is intermediate between the homozygous genotype groups, hence proposing an additive gene action for *Rpgaq1*(Figure 2B).

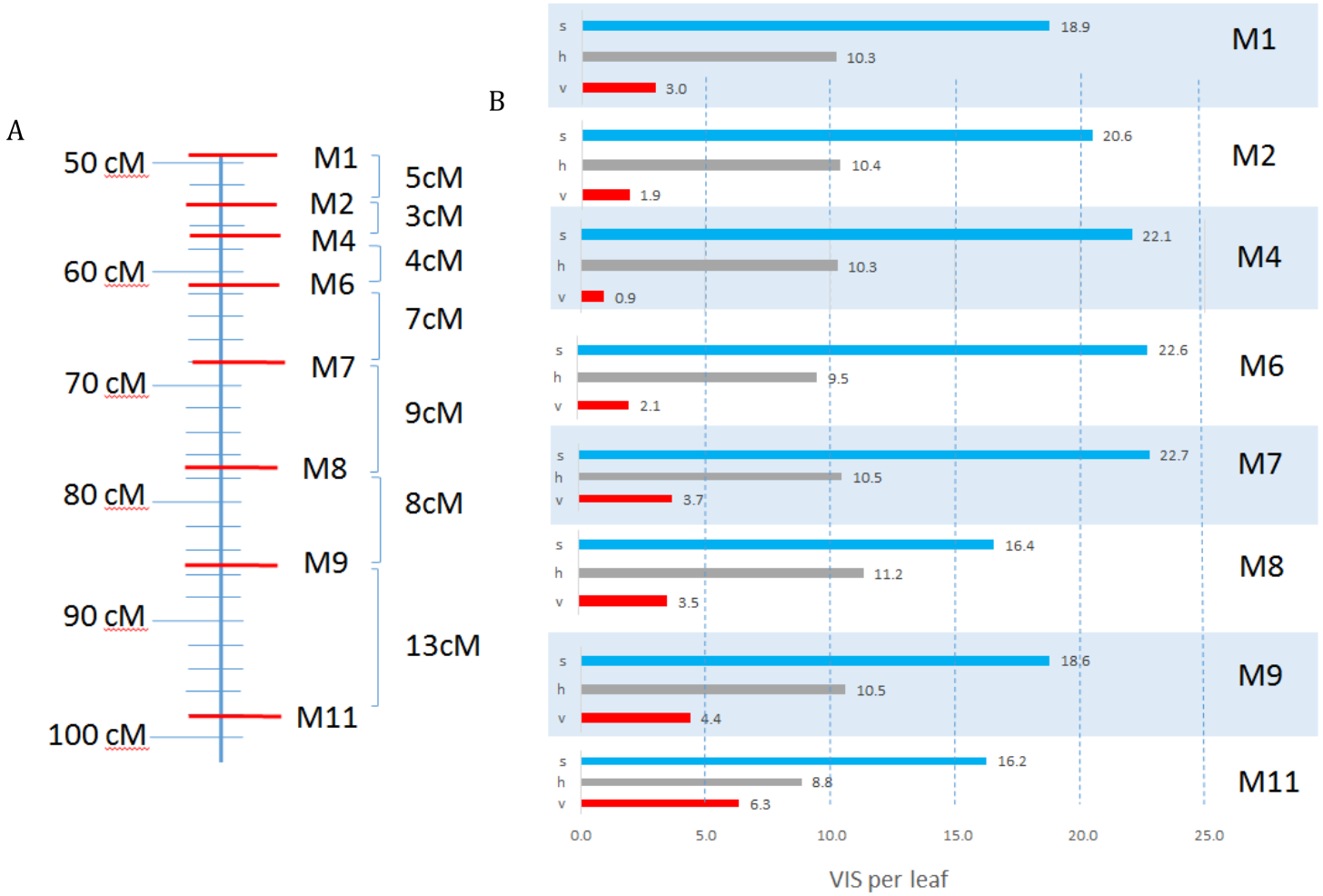


Figure 2: **A**: Barley VxS liLinkage map on 1H chromosome based on recombinations of F_2 Mapping population by Nansamba et al. (unpublished). **B**: average visual infection sites (VIS) per whole leaf per genotype group homozygous (s)usPtrit, (h)eterozygous, and homozygous(v)ada.

2.1.2.Fine mapping *Rpgaq5* based on F_2 population

In the *Rpgaq5* Nansamba (unpublished) identified ten SNPs in the target region around the LOD peak marker. Two SNPs (M15 and M23) were previously used to genotype the F_2 by Nansamba et al. (unpublished) while the remaining eight were tested for the presented study (Table 4).

We found that both M15 and M21 are only polymorphic after the annealing temperature was increased to 68°C (Table 4). Due to non-polymorphism between the parents, even after changing the annealing temperature, the following SNPs were unfit for further use: M14, M16, M18, and M20 (Table 4) Out of the four non-polymorphic SNPs, M18 was unexpected because it was reported to be polymorphic by Nansamba (unpublished) (Table 4). From the remaining six SNPs, M15 and M23 had already been genotyped by Nansamba, leaving only M17, M19, M21 and M22 for F_2 genotyping. Following a Chi-square Test to evaluate 1:2:1 segregation, the genotyping exposed segregation distortion in M19, favoring Vada alleles, and therefore excluded from further use (Table 4). Ultimately, we were left with three additional SNPs complementing the genotyping with M15 and M23 done by Nansamba (unpublished).

Marker	SNP ID	VxS consensus map (cM)	Nansamba Primer Test (at 60 C)	Confirmation	Chi-Square test
M14	BOPA1_7172-1536	7.5	Non-polymorphic	confirmed	
M15	**SCRI_RS_201028***	**6.0**	**Polymorphic**	**polymorphic at 68 °C**	**0.124**
M16	SCRI_RS_229445	N/A	Non-polymorphic	confirmed	-
M17	**SCRI_RS_207095**	**6.9**	**Polymorphic**	**confirmed**	**0.158**
M18	SCRI_RS_160297	10.6	Polymorphic	Non-polymorphic	-
M19	SCRI_RS_12396	10.6	Polymorphic	confirmed	0.001**
M20	SCRI_RS_172655	N/A	Non-polymorphic	confirmed	-
M21	**SCRI_RS_13615**	**10.6**	**Polymorphic**	**polymorphic at 68 °C**	**0.067**
M22	**SCRI_RS_230959**	**11.2**	**Polymorphic**	**confirmed**	**0.095**
M23	**SCRI_RS_42792***	**11.8**	**Polymorphic**	**confirmed**	**0.112**

Table 4: SNPs tested on parents and mapped in *Rpgaq5* mapping population to confirm polymorphism previously tested by Nansamba (unpublished). Bold rows were used to genotype F_2, non-bolded rows contain abandoned SNPs. *SNP used by Nansamba to genotype. 1:2:1 segregation was tested with Chi-Square test: ** significantly different ($p<0.01$). All SNPs were tested at 60°C melting temperature unless indicated otherwise.

The linkage map calculation produced a 3 cM linkage map, based on the information from 70 plants (Figure 2A). Next, we calculated the averaged VIS per genotype group (v, h, and s) which showed that homozygous Vada individuals were considerably more susceptible than the homozygous SusPtrit and heterozygous genotype groups, as expected for the resistance donor SusPtrit (Figure 3B). The similar infection levels between homozygous SusPtrit and heterozygous geneotype groups suggest a dominant gene action of *Rpgaq5* (Figure 3B). Unfortunately, it was not possible to fine map *Rpgaq5* further by comparing the averaged VIS per genotype group across all SNPs (Figure 3B). Because the markers already locate to a narrow interval, a simple comparison of the genotype groups between markers will not demonstrate large phenotypic differences. Hence, heterozygous recombinants need to be tested in inoculation trials to fine map *Rpgaq5* further. Additionally, the recombination frequencies between M21-M23 show that they inherit together (Appendix 3B). The most reliable marker was M21 and therefore chosen as the right flanking marker, along with M15 as the left flanking marker for future experiments.

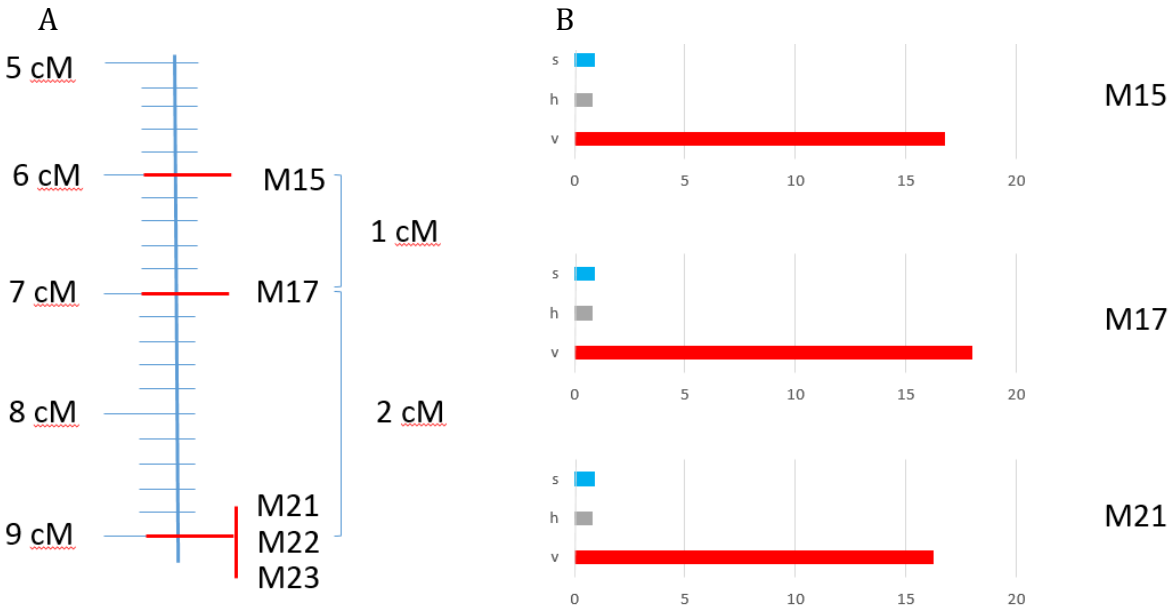


Figure 3 A: Barley VxS linkage map containing *Rpgaq5* on 7H chromosome based on recombinations of F_2 Mapping population with 70 plants. **B:** average visual infection sites (VIS) per whole leaf per genotype group homozygous (s)usPtrit, (h)eterozygous, and homozygous (v)ada.

2.2. Observing phenotypic resistant/genetically susceptible individuals in disease tests

By studying the F_2 phenotyping results for the 7H target region of Nansamba et al. (unpublished), we noticed a high frequency of (relatively) resistant homozygous Vada genotypes which were expected to have a susceptible phenotype (Table 5). Therefore, we tested if the absence of infection in individual plants is full poof that they are not genetically susceptible (Table 5). The test was done in the *Rpgaq5* mapping population but the results should be applicable beyond.

Overall, 6 of 24 homozygous Vada families demonstrated low VIS (between 0 and 2 VIS per leaf) (Appendix 2). Essentially showing that 25% of the overall homozygous Vada averaged 1.2 VIS per leaf compared to an average 17.4 VIS per leaf of all 24 homozygous Vada families. We tested three relatively resistant homozygous Vada families and observed that the progeny was susceptible with more than 5 pustules per leaf (Table 5). This suggests that genetically susceptible plants are not guaranteed to have a susceptible phenotype, but can seem resistant in some cases. This could be conditioned by the experimental error, such as inhomogeneous spore distribution or germination.

Sample	VIS	M15	M21	Progeny
2.16	1	VV	VV	Fully susceptible
2.56	0	VV	VV	Fully susceptible
2.44	2	VV	VV	Fully susceptible

Table 5: Observing the progeny of susceptible parents with an unusually low VIS.

2.3. Recombinant screening and continued linkage mapping

For improved resolution of the newly identified target regions, flanking markers of the *Rpgaq1* and *Rpgaq5* mapping populations, were used to genotype the progeny of heterozygous F_2 individuals to identify recombinations. Saturating the target regions with recombinants is essential for seedling tests to elucidate the target regions harbouring *Rpgaq1* and *Rpgaq5*. Unfortunately, it was not possible to use the described phenotyping of susceptible recombinants because unreliable scoring rendered the results useless. However, the identified recombinants and the updated linkage map (including new SNPs) were essential for the continued fine mapping of *Rpgaq1* and *Rpgaq5*.

2.3.1. *Rpgaq1* recombinant screening and SNP development

Considering the 3 cM interval for *Rpgaq1*, we chose M2 and M6 to screen for recombinants in the progeny of fully heterozygous F_2 individuals. Overall, nine families (581 individuals) were screened. Out of nine families, one family (2.21) was not fully heterozygous but was fully Vada for M2 (Appendix 6A). Incorrect genotyping in the F_2 could be responsible. From 506 screened individuals we identified 22 sh recombinants and 25 vh recombinants (Appendix 7 and 8). (For the difference between sh and vh recombinants see Table 2 in Materials and Methods)

	Marker	SNP ID	VxS SNP. Map position	Forward and Reverse Primers
A	J1	SCRI_RS_170869	65.9	F:AAGTTTGTGCAGGAGGTGGT R: CATGATCTGGGAGCAGTCGG
	J2	SCRI_RS_189483	66.5	F:GCAAGCTATGGATCAGCTGC R:GGGCACAAACTGAAGCTCATC
Rpgaq1	J3	SCRI_RS_189637	66.5	F: CCAGTTTTTCGGGGATGG R:ATCAAGGGAATAGTCGTTTGGTG
	J4	SCRI_RS_187264	67.1	F:TGGACATTTGTGTCTATGTTTTTCT R:CTGGGATATACATAGATTAAGTGTCAA
	J5	SCRI_RS_147042	67.1	F:TGCAAAGTACATCATGAAAACAGAT R:GTTGCTCTTCTTCAGGCTAGACAT
B	J6	SCRI_RS_194557	7.5	F:AGATACTGATAAAATTGATATCCTAGA R:TAGCAAAACTTGTCTGGACCCTC
	J7	BOPA1_1555-631	7.5	F:TGGTTGATTACAAACTGATCT R:TAAAAATTATGGAGTCCACTG
Rpgaq5	J8	SCRI_RS_12396	10.6	F:GGTAGAAACATACACAAAGTTGTACT R:CCACTTATTTTGGGACGGAGG

Table 6: Polymorphic SNPs identified in barley mapping cross Vada x SusPtrit relevant for the elucidation of chromosome intervals A: *Rpgaq1* and B: *Rpgaq5*.

Following the recombinant screening we improved the resolution of the target region, by developing new primers for five additional SNPs showing polymorphic segregation (Table 6A). Following genotyping, a F_3 linkage map was created based on 506 individuals and therefore a higher distance reliability compared to the F_2 linkage map (Figure 4). Using the new SNPs, it was possible to increase the resolution of the point of recombination for each recombinant. Also, future disease tests can rely on a higher SNP saturation of the target region, which will allow closer fine mapping of *Rpgaq1*. Recombination frequencies suggested that J2, J3 and J4 commonly inherit together with only one recombinant identified between each marker pair (Figure 4, Appendix 4A). Overall, J2 showed the highest reliability between the three closely linked SNPs.

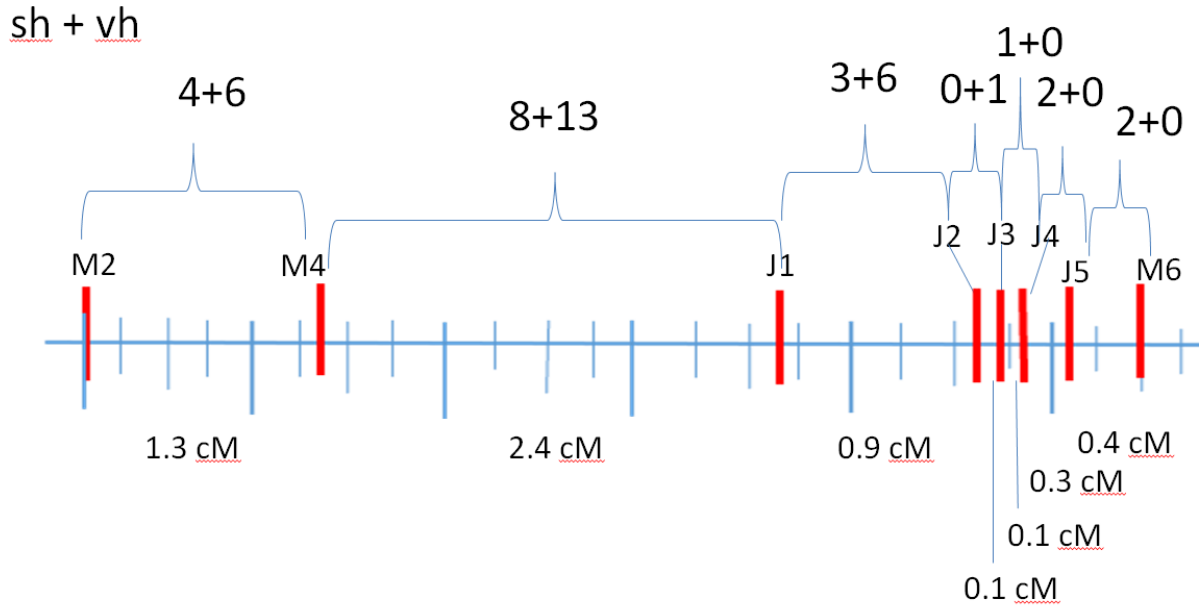


Figure 4: 1H chromosome linkage map including the number of identified recombinants (sh+vh) differentiated into heterozygous recombinants with a SusPtrit stretch (sh) and heterozygous recombinants with a Vada stretch (vh). cM distances are based on observed recombination frequencies.

2.3.2. *Rpgaq5* Recombinant Screening and SNP development

For *Rpgaq5* we chose M15 and M21 to screen eleven families of fully heterozygous F_2 individuals (434 individuals). One family (2.42.) was not genotyped correctly in the F_2 because M15 was homozygous SusPtrit in the progeny (Appendix 6B). This left 404 individuals, which were screened for recombinations between M15 and M21, this identified 16 vh recombinants and 18 sh recombinants (Appendix 9 and 10). Next, new primers were developed for three polymorphic SNPs identified on the VxS SNP map and used to improve the resolution of the 3 cM region of interest (Table 6B). Notably, SNP SCRI_RS_12396, previously M19 (Chi Sq, p=0,001)(Appendix 2), was successfully run as J8 using new primers (Table 6B).

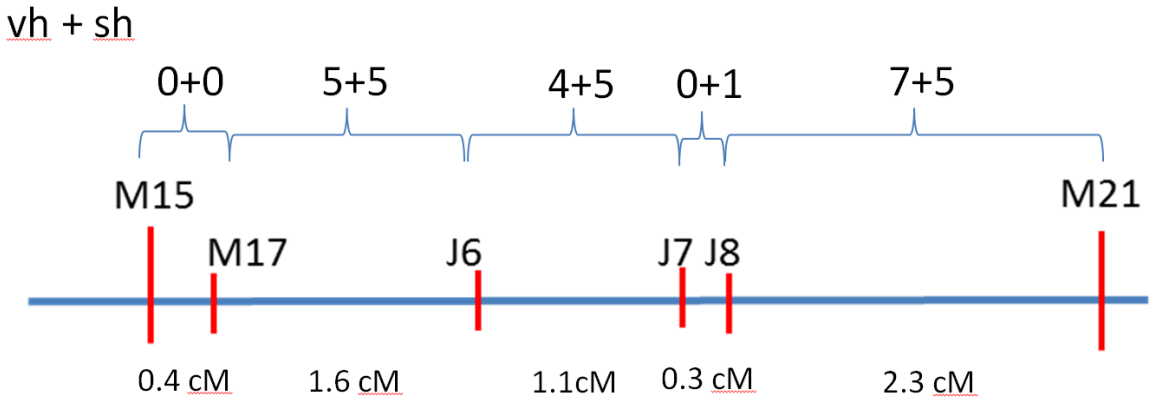


Figure 5: 7H chromosome linkage map containing *Rpgaq5*, including identified recombinants (vh+sh). cM distances are based on observed recombination frequencies. Two sh recombinants were omitted because the exact location of recombination is unknown due to missing data (Appendix 10).

Based on the recombination frequencies between the genotyped SNPs, distances were calculated and a linkage map was developed based on 404 individuals, resulting in higher distance reliability and SNP saturation compared to the F_2 linkage map (Figure 5). We did not find recombinantions between M15 and M17 (Appendix 10). However, we identified a vh recombinant (2.69) in the F_2 material by Nansamba et al. (unpublished)(Appendix 2). This family was added to the promising material for seedling testing, identified in the presented heterozygous screening.

2.4. 1st Seedling Test

From F_2 seed, *Rpgaq1* and *Rpgaq5* recombinant families were grown and phenotyped following a disease test with *Pga* Evertsholm. However, for this seedling test phenotyping only considered the quantification of pustules and did not account for flecks (>0.5 mm). The phenotyping is however not sufficient because flecks also deem individuals susceptible. Therefore, what might seem like a resistant individual, if only based on the absence of pustules, may be a misclassification of a susceptible individual due to unrecorded flecks. Hence, we only considered resistant recombinants for the fine mapping because if the QTL maps to the homozygous stretch we expect a resistant family but a segregating family if it maps to the heterozygous stretch. Therefore, if >5 pustules appear in ca. 25% individuals of the family we can assume a 3:1 segregation for the susceptible genotype of the QTL, and therefore map it in the heterozygous stretch of the parental recombinant. For the mapping populations *Rpgaq1* and *Rpgaq5* the resistant heterozygous recombinants were vh and sh recombinants respectively.

2.4.1.Rpgaq1 position

For *Rpgaq1* seven recombinant families were tested and genotyped with M2 and M6. Families 2.38 and 2.49 showed the segregating phenotype mapping the QTL to the heterozygous stretch of the parental recombinant. This allowed the fine mapping of *Rpgaq1* between M1 and M7 (Table 7). Essentially, >5 infections were observed, on 17% and 25% of the individuals of families 2.38 and 2.49 respectively (Figure 6). Somewhat lower infection frequencies than 25%, could be attributable to the reduced reliability of overall phenotyping. The families are presented according to their genotype groups and the corresponding average number of pustules per leaf (Figure 6). Both families show significant differences between vv and ss genotype groups according to a Student's T test ($p<0.05$), therefore promoting the claim that both families are segregating for resistance (Appendix 11). The Chi-Square Test suggested that the expected 1(vv):2(hh):1(ss) segregation does not differ significantly for 2.38 and 2.49 respectively (Table 7). Overall, it was not possible to increase the resolution of *Rpgaq1*, however the fine mapping results from the F2 genotyping were confirmed.

Sample	F_2 VIS	M1	M2*	M4	M6*	M7	M8	Progeny	Chi-Sq.
2-38	1	h	h	h	h	v	v	**Seg**	X^2=0.93
2-32	1	h	v	v	v	v	v	Unknown	
2-61	0	v	v	v	h	h	h	Unknown	
2-49	15	v	h	h	h	h	h	**Seg**	X^2=0.33
2-12	1	s	s	h	h	h	h	Unknown	
2-5	14	h	s	s	s	s	s	Unknown	
2-69	14	h	h	h	h	h	s	Unknown	

Table 7: Genotypes of F_2 individuals selected for family seedling test with *Puccinia graminis* f. sp. Avenae isolate Evertsholm elucidating 1H chromosome. Progeny was phenotyped for Pustules per leaf and evaluated for segregation. Progeny with unknown resistance, susceptibility or resistance due to incomplete phenotyping were not considered reliable (presented as unknown). *denotes tested SNPs. Chi-Square Test ($p<0.05$) was conducted to confirm an expected 1(vv):2(hh):1(ss) allele segregation of tested families.

Also, it is noteworthy that while 2.32 and 2.61 were not phenotype reliably, no pustules were observed in either family, hinting at fully resistant families (Table 7). This is not conflicting with the results of 2.38 and 2.49. Overall, we used this insight to design additional experiments, which might give more reliable evidence to the proposed QTL location. Furthermore, against expectation we did not witness an additive gene action, previously observed during F_2 genotyping. An explanation for this could be the limited conclusiveness of the phenotyping because especially the intermediate susceptible phenotypes might have been observed as resistant rather than susceptible. However, other factors such as segregating QTLs in the background of the mapping population could also be responsible.

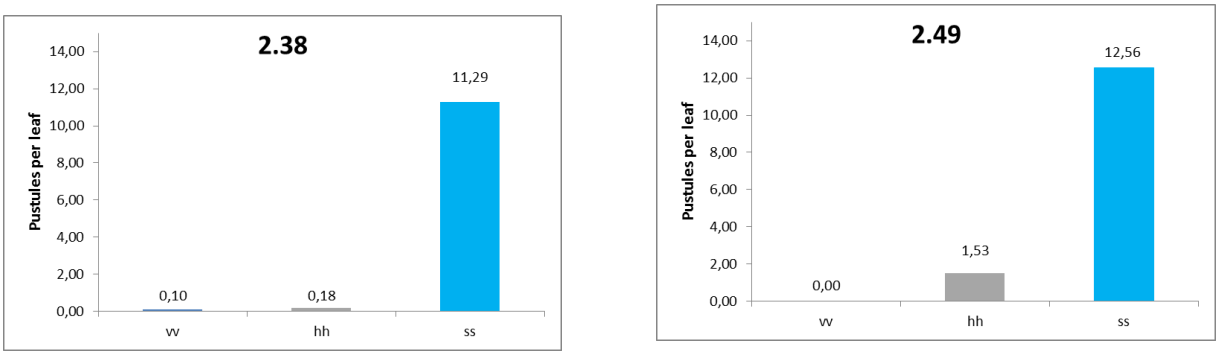


Figure 6: The average number of pustules per leaf according to the genotype groups (vv, hh, and ss) for 1H family 2.38 and 2.49, following a seedling test with *Puccinia graminis* f. sp. *avenae* isolate Evertsholm.

2.4.2.Rpgaq5 position

For the *Rpgaq5*, three recombinant families were genotyped with M15 and M21. This included one family of a resistant recombinant (2.70); the family showed >5 pustules in 8% of the individuals of the family, mapping the QTL left of M21 (Table 8). The 8% infection compared with the expected 25% infection in the family seem too low. However, it will be reoccurring that the infection frequency of the *Rpgaq5* mapping population is lower than the infection frequency of the *Rpgaq1* mapping population at the same spore density. Hence, the low frequency (8%) of susceptible individuals (>5 pustules) could mean that the infection level was too low, to expose all genetically susceptible individuals, phenotypically. In any case, we will use this experiment to give additional weight to the previous results but also plan additional seedling tests for increased reliability.

Sample	F_2 VIS	M15	M17	M21	Progeny	Chi Sq.
2.70	1	h	h	ss	**Segregates**	X^2= 0.6
2_53	0	h	h	v	Unknown	
2_69	1	v	h	h	Unknown	

Table 8: Genotypes of F_2 individuals selected for family seedling test with *Puccinia graminis* f. sp. Avenae isolate Evertsholm elucidating 7H chromosome. Progeny was phenotyped for Pustules per leaf and evaluated for segregation. Chi square Test conducted to confirm 1(v):2(h):1(s) allele segregation.

Using M17, the 2.70 family demonstrated strong tendencies for significant differences between genotype groups ss and vv (p=0.06)(Appendix 12). This confirmed the segregating character of the presented family (Figure 7). The observed segregation did not differ significantly to the expected 1:2:1 segregation (Table 8). Overall, it was possible to confirmed the previous results that also placed the QTL left of M21 (Table 8). And show that the presented data has a dominant gene action, which confirms results from the F_2 genotyping.

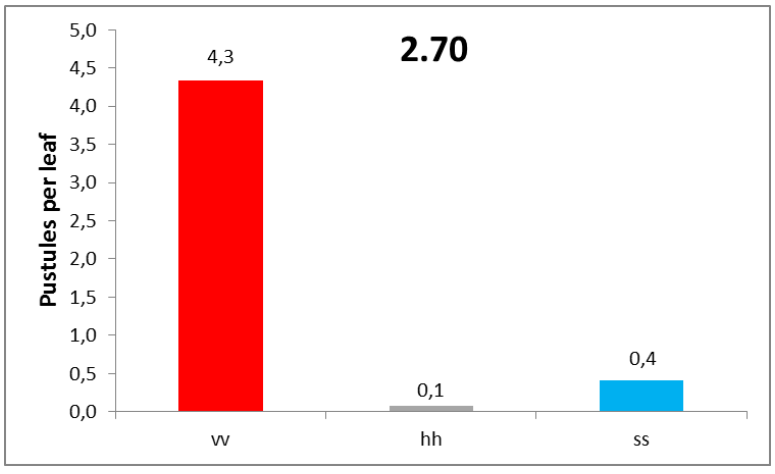


Figure 7: 7H family 2.70 mapping *Rpgaq5* showing the average number of pustules per leaf according to the genotype groups (vv, hh, and ss) for, following a seedling test with *Puccinia graminis* f. sp. Avenae isolate Evertsholm.

2.4.3. Conclusions for 1st seedling test

While, this experiment is generally considered to be a failure it was possible to extract several valuable insights about possible QTL locations. However, the insights of this experiment are not conclusive due to the unreliable nature of the phenotyping. Nevertheless, the results do not contradict our previous results from the fine mapping of the F_2 genotypes. In addition, it was possible to collect several homozygous recombinants in the 1st seedling test, which might be valuable at a later stage (Appendix 13A+B). Additional experiments were designed to continue to elucidate the position of both *Rpgaq1* and *Rpgaq5*.

2.5. 2nd Seedling test

From available seed of selected recombinants, identified in the recombinant screening, we selected 4 families per QTL and performed a seedling test on the progeny of each (Appendix 14). Overall, the infection levels were not very high, even though the control oat plants showed heavy infections. It is unclear if the utilized frozen spores were more virulent on host plants compared to near non-host plants. In any case, we expected a similar infection level between the oat controls and the homozygous susceptible genotype groups. However, this was not confirmed by the seedling test. Nevertheless, the phenotyping was done to attempt to narrow down the target region for *Rpgaq1* and *Rpgaq5* (Appendix 15 and 17). However, comparing genotype and phenotype results did not allow differentiation between segregating families and fully susceptible families. Hence, we could not extract valuable information from this seedling test. Therefore, we devised a follow up: our 3rd seedling test. However, homozygous recombinants identified in the 2nd seedling test were transplanted to set seed (Appendix 13).

2.6. Confirmation of QTL location

After the unsuccessful 2nd seedling test we were not confident that the conclusions drawn from previous results were convincing enough to expect each QTL in the previously proposed intervals. Hence, we tested progeny of homozygous recombinants (identified during the 1st seedling tests) for the proposed target region of each QTL and inoculated 10 seedlings with *Pga* Evertsholm to confirm our previous results.

For the *Rpgaq1* target region we mapped the QTL position between M2 and M6 using individuals from homozygous recombinant families with reciprocal genotypes (Table 9). All individuals were resistant to *Pga* Evertsholm. For *Rpgaq1* the resistance donor is Vada and therefore we can conclude that *Rpgaq1* is to the right of M2 and to the left of M6 (Table 9). Therewith, confirming the previously proposed chromosome region.

Sample	M2	M4	M6	Phenotype
2.12.10	ss	vv	vv	Fully Resistant
2.61.26	vv	vv	ss	Fully Resistant

Table 9: *Rpgaq1* homozygous recombinants inoculated with *Pga* Evertsholm. The phenotyping was done visually, distinguishing between fully susceptible or fully resistant families.

For *Rpgaq5* we inoculated three homozygous recombinants for the target region with *Pga* Evertsholm (Table 10). However, 2.12.15 is not a homozygous recombinant but a falsely genotyped heterozygous individual resulting in segregating progeny. In any case, both of the homozygous recombinants were used to confirm the previously proposed target region. For *Rpgaq5*, SusPtrit is the resistance donor producing resistant families. Therefore, fully resistant 2.69.27 suggests that *Rpgaq5* is to the right of M15. While, fully susceptible 2.70.8, suggests that the target region is to the left of M21 (Table10). Therefore, we can confirm the previously suggested target interval between M15 and M21.

Sample	M15	M17	M21	Phenotype
2.69.27	Vv	ss	ss	Fully Resistant
2.70.8	Vv	vv	ss	Fully Susceptible
2.12.15	Hh	hh	hh	Segregates

Table 10: *Rpgaq5* homozygous recombinants inoculated with *Pga* Evertsholm. The phenotyping was done visually, distinguishing between fully susceptible or fully resistant families.

This small but conclusive experiment established a reliable groundwork for continued fine mapping work in both QTL intervals. Next, we identified informative recombinants for each target region from a pool of recombinants identified during the recombinant screening, set to be tested in an additional seedling test (Appendix 7-10).

2.7. 3rd Seedling test

Similar to all previous seedling tests we sowed heterozygous recombinants per SNP interval of each mapping population, and phenotyped the progeny to infer the QTL location (Table 11 and Table 15). To allow the fine mapping we determined trait segregation or full susceptibility of families, and compared the outcomes between families.

2.7.1. Determining segregating and fully susceptible families for *Rpgaq1*

We tested seven sh families with a recombination for each marker interval. Moreover, for each family the average VIS/cm^2 was compared between homozygous susceptible and homozygous resistant genotype groups for each segregating SNP (Figure 8). A distinction between segregating and fully susceptible families is done with a comparison of means according to Student's test ($p<0,05$) (Table 12). If there are no significant differences for the infection frequency between both homozygous genotype groups then the family is considered fully susceptible or fully resistant. (Table 12). However, if significant differences are reported between both homozygous genotype groups we consider the family to segregate (Table 12). Determining if progeny was segregating or fully susceptible mapped the QTL position to the homozygous or heterozygous stretch of parental recombinants (Table 11).

	SNP Markers								Progeny
Sample ID	**M2***	**M4**	**J1**	**J2**	**J3**	**J4**	**J5**	**M6***	
2.14.10	ss	hh	hh	hh	hh	hh	hh	hh	Segregates
2.54.48	ss	ss	hh	hh	hh	hh	hh	hh	Segregates
2.54.59	**ss**	**ss**	**ss**	**hh**	**hh**	**hh**	**hh**	**hh**	**Segregates**
2.14.24	**ss**	**ss**	**ss**	**ss**	**hh**	**hh**	**hh**	**hh**	**Full Suscept**
2.54.38	ss	ss	ss	ss	ss	ss	hh	hh	Full Suscept
2.43.34	ss	ss	ss	ss	ss	ss	ss	ss	Full Suscept
2.14.16	hh	hh	hh	hh	hh	ss	ss	ss	Segregates

Table 11: Heterozygous recombinants proposed for disease testing with *Pga* Evertsholm to elucidate 1H target region using 8 SNP Markers in barley mapping population VxS. SNP locus: ss= homozygous SusPtrit, hh= heterozygous. Bold families are especially helpful to fine map *Rpgaq1*. * indicates the proposed SNP used for genotyping recombinant family

Three families were declared fully susceptible following the determination of the average VIS/cm^2 per genotype group and a subsequent a mean comparison (Table 11 and Table 12). Surprisingly, 2.43.34 was homozygous SusPtrit and did not segregate for M6 in the porgeny, and therefore the Student's T test was not performed between genotype groups (Table 12). Yet comparing the susceptible control RIL 152 and 2.43.34 progeny, did not expose significant difference between the average VIS/cm^2 (Table 12). Moreover considering that all individuals of the 2.43.34 family had a relatively high infection we are confident to regard it fully susceptible (Appendix 22).

Further, four families differed significantly for the average VIS/cm^2 between homozygous Vada and homozygous SusPtrit genotype groups and were therefore considered to segregate (Table 11 and Table 12). Contrary to the F_2 population we observed a dominance rate which, similar to the 1st seedling test, suggests a discrepancy between the F_2 dominance rate and later disease tests. Moreover, we found remarkable infection differences between the homozygous susceptible (SS) genotype group and the susceptible control (RIL 152) (Figure 8). Interestingly, some families showed significantly higher susceptibility than the susceptible control RIL 152, while others families where significantly lower or indifferent to the susceptible control (Table 12).

		Student's T Test		Chi-Square
Sample	**F_3 Progeny**	**VV and SS**	**SS and RIL 152**	**1:2:1**
2.14.10	Segregates	p<0.001*	p=0,844	X^2= 0.37
2.54.48	Segregates	p=0.002*	p<0,001*	X^2= 0.06**
2.54.59	Segregates	p<0.001*	p<0,001*	X^2= 0.51
2.14.24	Full Suscept	p=0.84	p=0,054**	X^2= 0.30
2.54.38	Full Suscept	p=0.88	p=0,024*	X^2= 0.90
2.43.34	Full Suscept	N/A	p=0,380	N/A
2.14.16	Segregates	p<0.001*	p<0,001*	X^2= 0.45

Table 12: Observations of F_3 progeny grouped according to segregating heterozygous marker (VV, HH and SS). The VIS/cm^2 of the homozygous Vada and homozygous SusPtrit groups, and the VIS/cm^2 of the heterozygous and homozygous Vada groups were compared using Student's T-Test. * significantly different mean (p<0.05); ** tendency for significant difference in 1:2:1 segregation tested with Chi square test.

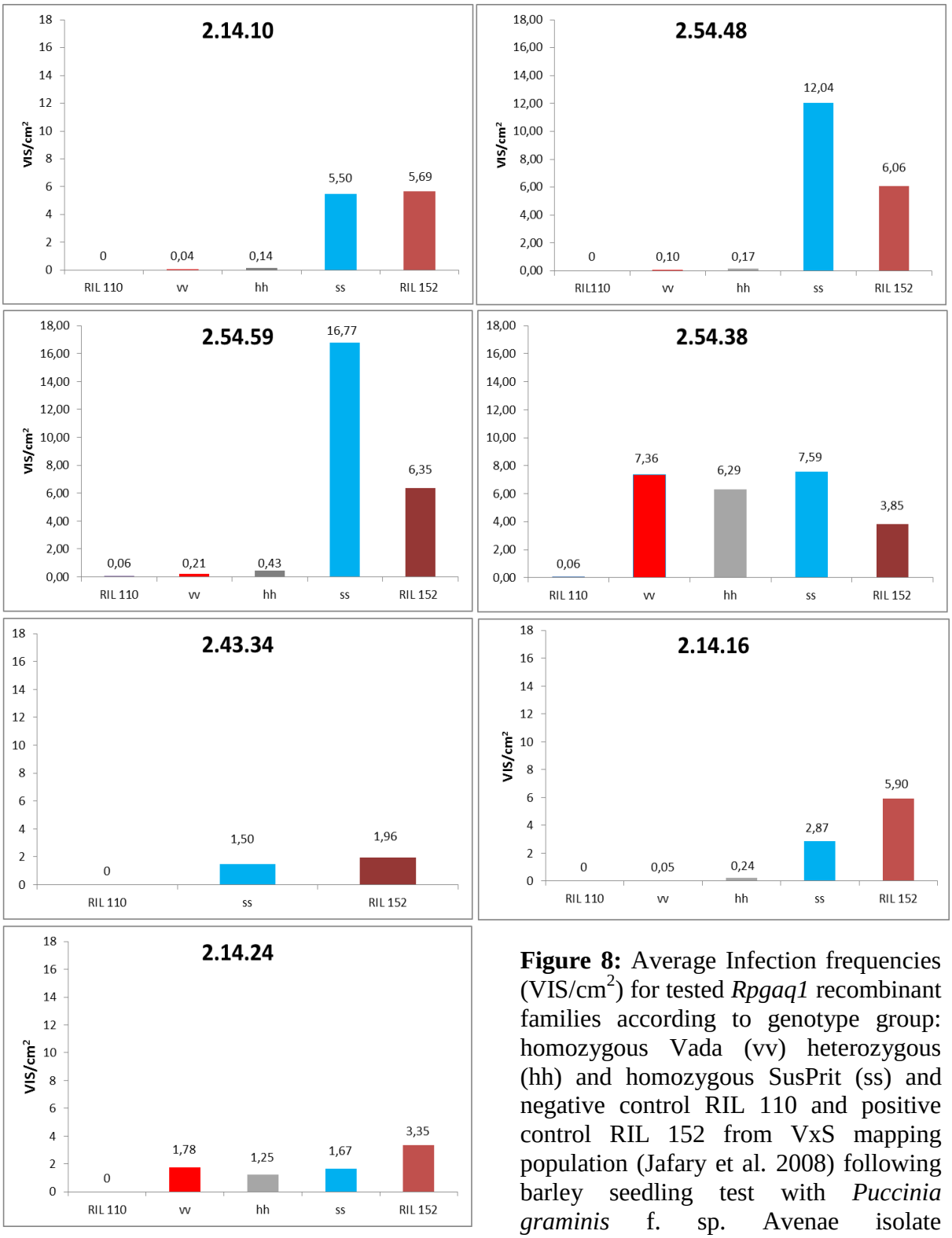


Figure 8: Average Infection frequencies (VIS/cm^2) for tested *Rpgaq1* recombinant families according to genotype group: homozygous Vada (vv) heterozygous (hh) and homozygous SusPrit (ss) and negative control RIL 110 and positive control RIL 152 from VxS mapping population (Jafary et al. 2008) following barley seedling test with *Puccinia graminis* f. sp. Avenae isolate Evertsholm.

Overall, segregating marker loci are expected to undergo a 1:2:1 allele segregation, and these expectations were tested using a Chi-Square Test (Table 12). The family 2.43.34 was not tested because all progeny are homozygous SusPtrit with no allele segregation. Only family 2.54.48 was close to the threshold statistically questioning random assortment (p=0.06) (Table 12) However, the large VIS/cm^2 differences between genotype groups do not suggest non-random assortment (Appendix 19). Hence, we are confident that there is no segregation distortion. Overall, especially the Families 2.14.24 and 2.54.59 have been informative to fine map *Rpgaq1* left of J3 and right of J1 respectively, to a 1cM interval (Table 11). In line with other seedling tests is the dominant gene action for *Rpgaq1* which contradicts findings during the F_2 genotyping.

From the F_3 heterozygous recombinant screening we identified 3 sh heterozygous recombinants and 7 vh heterozygous recombinants for the proposed target region between J1 and J3 (Table 13). Based on previous results we know that the interval between J1 and J2 is 0.9 cM and between J2 and J3 it is 0.1 cM (Figure 4). For the J2-J3 interval only one heterozygous recombinant is available. The seeds for all recombinants are archived and can be used for further linkage mapping once additional SNPs have been developed.

Sample	J1	J2	J3
2.18.2	s	h	h
2.54.59	s	h	h
2.27.8	s	h	h
2.60.36	v	h	h
2.60.59	v	h	h
2.27.37	v	h	h
2.14.24	v	v	h
2.58.2	h	v	v
2.60.47	h	v	v
2.58.51	h	v	v

Table 13: heterozygous individuals with recombinantion in 1cM interval identified to harbor *Rpgaq1*.

Further, it possible to differentiate visually between families with significantly higher susceptibility compared to the susceptible control, and families with no significant difference (or significantly lower susceptibility) compared the susceptible control (Figure 9). We noticed that families originating from F_2 individuals 2.54 and 2.14 (2.43.34 was not recorded) showed obvious differences in the infection frequencies between each other (Figure 9). We observed that the 2.54 origin produced a much higher ratio of teliospores to uredospores compared to the 2.14 origin (Figure 9). The visual infection difference was consistent across all trays if we compare it to the positive control RIL 152 (Figure 8). Generally, we expected that RIL 152 would always be the most susceptible line per tray. However, it became clear that families originating from F_2 individual 2.54 are significantly more susceptible than the susceptible control (Table 12). Therefore, we believe that the differences between the families have a systematic component, that could be conditioned by genetics. One explanation is that families of the mapping population might segregate for a minor QTL, unknown to affect *Pga* Evertsholm, for which the susceptible control (RIL 152) had the resistance allele conditioning a reduced susceptibility compared to the homozygous susceptible genotype group of 2.54 families.

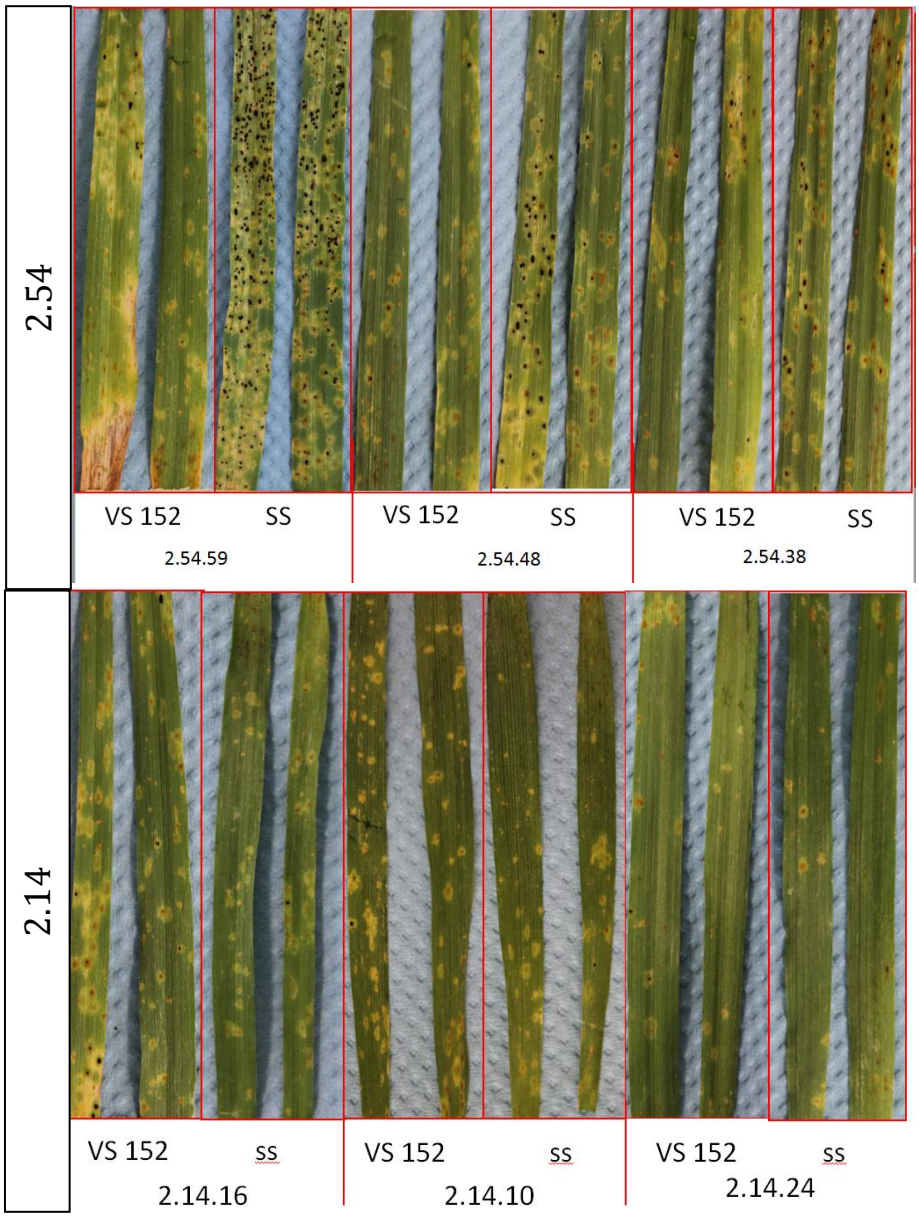


Figure 9: Infection levels of random individuals from the positive control RIL 152 and the susceptible ss genotype group presented according to tested *Rpgaq1* families. On top families originating from F_2 individual 2.54. On bottom families originating from F_2 individual 2.14.

For improved comparability between families, we calculated a ratio of the average VIS/cm^2 between the susceptible genotype group (ss) and the susceptible control RIL 152. Therefore, adjusting for the differences in the infection frequency between tested trays (Table 14). Forming the ratios, shows that grouping the families according to their F_2 origin (2.54, 2.14 and 2.43) helps to distinguish the families according to their degree of susceptibility (Table 14). Especially, the adjustment of 2.54.38 helps to associate it with the remaining 2.54 families, all together showing a ratio ≥ 2 (Table 14). This also applies for 2.43.34, which can be associated better to 2.14 families, with ratios ≤ 1 (Table 14).

F_2 Origin	**Tested 1H families**	**Average VIS/cm^2**		**Ratio**
		ss Group	**VS 152**	**ss/152**
2.54	2.54.48	12,04	6,06	2,0
	2.54.59	16,77	6,35	2,6
	2.54.38	7,59	3,85	2,0
2.43	2.43.34	1,50	1,96	0,8
2.14	2.14.16	2,87	5,90	0,5
	2.14.24	1,67	3,35	0,5
	2.14.10	5,50	5,69	1,0

Table 14: The average VIS/cm^2 of individuals in the ss genotype group of *Rpgaq1* families, and the positive control RIL 152. Ratio between average VIS/cm^2 of individuals in SS genotype group and RIL 152 to adjust for differences in the infection frequency.

2.7.2.Determining segregating and fully susceptible families for *Rpgaq5*

The third seedling test for *Rpgaq5*, consisted of five families with a recombination for each marker interval (Table 15). The heterozygous SNP in each family was used to genotype the five families. Next, the average VIS/cm^2 was calculated for each genotype group, and also for the susceptible and resistant RIL control (Figure 11). Those families segregating for resistance showed a significant differences between the homozygous genotype groups (Table 16). The fully susceptible families on the other hand did not show significant differences between the homozygous genotype groups (Table 16)

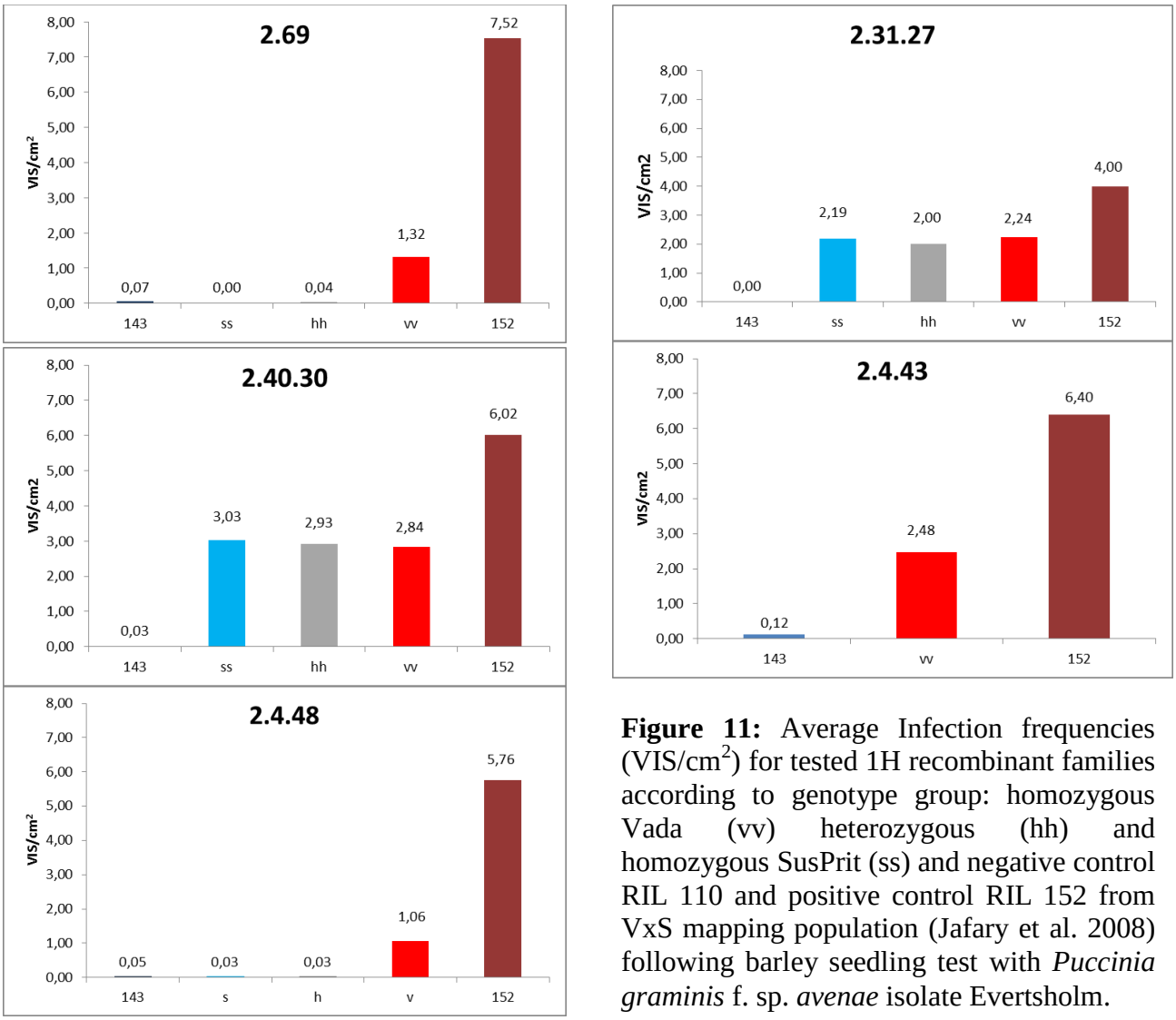


Figure 11: Average Infection frequencies (VIS/cm^2) for tested 1H recombinant families according to genotype group: homozygous Vada (vv) heterozygous (hh) and homozygous SusPrit (ss) and negative control RIL 110 and positive control RIL 152 from VxS mapping population (Jafary et al. 2008) following barley seedling test with *Puccinia graminis* f. sp. *avenae* isolate Evertsholm.

	SNP Markers						Family Phenotype
Family ID	M15	M17	J6*	J7	J8	M21*	
2.69	vv	hh	hh	hh	hh	hh	segregates
2.31.27	vv	vv	hh	hh	hh	hh	Full Suscept
2.40.30	vv	vv	vv	vv	hh	hh	Full Suscept
2.4.43	vv	vv	vv	vv	vv	vv	Full Suscept
2.4.48	hh	hh	hh	vv	vv	vv	segregates

Table 15: Informative heterozygous recombinants proposed for disease testing with *Pga* Evertsholm to elucidate 7H target region using 6 SNPs in barley mapping population VxS. Asterisk (*): SNPs J6 and M21 were used to elucidate locus heterozygous in parent: vv= homozygous Vada, hh= heterozygous; identified families segregating for resistance based on average VIS/cm^2 per genotype group (Figure 10).

We found three fully susceptible families (Table 15). To confirm full susceptibility, a Student's T-test was performed if possible (i.e. familiy 2.40.30) otherwise, we relied on the phenotypes of the tested families (Appendix 26 and 28). For the family 2.40.30, no significant differences were found between average means of both homozygous genotype groups, therefore the family is considered fully susceptible (Table 15). For both other families it was not possible to conduct a Student's T-test to statistically prove full susceptibility for the following reasons: 1) Genotyping the progeny of the tested families revealed that 2.4.43 is homozygous Vada for the target region, without a recombination between J8 and M21 (Appendix 9). Hence, it was not possible to conduct a mean comparison between the genotype groups. 2) Conducting a Student's T-test for 2.31.27 was not possible because only 1 individual was identified for the ss genotype group (Appendix 26). However, the single homozygous ss individual did not contradict the Chi-Square test (p=0.18) because only 15 individuals were phenotyped if full segregation was obvious by visual observation. Based on susceptible phenotype of all 15 tested individuals for 2.4.43 and 2.31.27 we concluded that the families are fully susceptible (Appendix 26 and 28).

Sample	F_3 Progeny	SS and VV	RIL 152 and VV	Chi-Square
2.69	Segregates	p<0.001*	p=0.006*	0.21
2.31.27	Full Suscept	N/A	p=0.059	0.18
2.40.30	Full Suscept	p=0.75	p=0.017*	0.22
2.4.43	Full Suscept	N/A	p=0.033*	N/A
2.4.48	Segregates	p=0.001*	p=0.031*	0.29

Table 16: Observations of F_3 progeny grouped according to segregating heterozygous marker (VV, HH and SS). The average VIS/cm^2 of the homozygous SusPtrit and homozygous Vada groups, and the VIS/cm^2 of homozygous Vada compared to RIL 152 using Student's T-Test. * significantly different mean (p<0.05); ** significant difference between the expected and the observed segregation.

Families 2.69 and 2.4.48 are both considered to segregate, because the Student's T-test showed significant differences between both homozygous genotype groups (Table 16). Across all tested families, we did not witness segregation distortion; observations were not significantly different to the expected 1:2:1 segregation (Table 16). The most informative families are segregating family 2.69, mapping *Rpgaq5* right of M15, and fully resistant family 2.31.27, mapping *Rpgaq5* left of J6 (Table 15). Hence, the location of *Rpgaq5* was inferred and mapped to a 2 cM interval between M15 and J6 (Table 15). Furthermore, the dominance rate of *Rpgaq* 5 was confirmed, considering that we did not observe marked differences between homozygous individuals and heterozygous individuals (Figure 11). Contrary to *Rpgaq1*, a consistent dominance rate for *Rpgaq5* confirmed results from the F_2 genotyping and the first seedling test.

From the recombination screening and the previous seedling tests there are 11 heterozygous and 5 homozygous recombinants available for the 2cM target region (Table 18). The homozygous recombinants have only been genotyped for the flanking markers M15 and J6 (Table 18). Especially the 1.6 cM interval between markers M17 and J6 needs to be saturated with additional SNPs for further fine mapping in the future.

Sample	M15	M17	J6
2.31.27	v	v	h
2.11.2	v	v	h
2.40.1	h	h	v
2.11.7	h	h	v
2.19.2	h	h	v
2.5.27	h	h	v
2.5.33	h	h	s
2.19.30	s	s	h
2.1.15	s	s	h
2.40.5	s	s	h
2.4.47	s	s	h
2.40.1.1	s	Unknown	v
2.40.1.3	s	Unknown	v
2.40.1.4	s	Unknown	v
2.11.7.4	s	Unknown	v
2.11.7.12	s	Unknown	v

Table 18: heterozygous and homozygous individuals with a recombination in the 1cM interval identified to harbor *Rpgaq5*.

Moreover we made the observation that opposite to *Rpgaq1*, all *Rpgaq5* families seem to have a significantly lower infection than the susceptible control ($p<0.05$), except 2.31.27 which only has a tendency to be significantly different ($p<0.1$) (Table 16). Additionally there seems to be variation for the degree of susceptibility in the homozygous Vada genotype group (Figure 11). To expose this variation, we calculated ratios between the average VIS/cm^2 for homozygous Vada and RIL 152 (Table 17). It also shows various infection levels of the families, all with a lower infection compared to RIL 152 (Table 17).

Sample	VV Group	RIL 152	VV/ RIL 152 Ratio
2.69	1.3	7.5	0.2
2.31.27	2.2	4.0	0.6
2.40.30	2.8	6.0	0.5
2.4.43	2.5	6.4	0.4
2.4.48	1.1	5.8	0.2

Table17: The average VIS/cm^2 of individuals in the VV genotype group of *Rpgaq5* families, and the positive control RIL 152. Ratio between average VIS/cm^2 of individuals in VV genotype group and RIL 152 to adjust for differences in the infection frequency between trials.

2.8. Adult Plant Test

Across all tested species, the growth rates were similar and the developmental stages of the plants did not differ beyond two positions on the zadoks scale. Cebeco Oat and Alfred Oat were evaluated as controls to demonstrated a typical disease progression. One Wheat accession was also tested for susceptibility to *Pga* isolate Ingeberga, however it remained immune during all developmental stages.

The pustules on the leaves of Cebeco and Alfred Oat controls showed the tendency to increase or stagnate on the highest infection level observed, during seedling growth and tillering (10 d and 24 d) (Figure 12). However, later we notice a decrease of the leaf infection during stem extension and heading (37 d and 51 d) (Figure 12) This trend continues during anthesis (65 d) and ends in absent leaf infection during maturing (79 d), while the stem infection frequency increased with increasing development stages (Figure 13).

The Barley accessions all exhibit the highest leaf infection during the seedling growth (10 d), and all barley accessions showed a reduced leaf infection in the tillering phase (24 d) except VxS 152, which did not get infected in developmental stages past the seedling stage (10d) (Figure 12). During stem extension (37 d) only L94 showed leaf infection while all other barley accessions did not (Figure 12). Later developmental stages did not show any leaf infection for any barley accessions (Figure 12A). Contrarily to the oat controls, the barley accessions developed teliospores as significant forms of leaf infection. Also the size of the pustules observed on barley were considerably smaller than the size of the oat pustules (Figure 12). Leaf infection was only relevant for developmental stages up to heading (51d), because heading and more advanced developmental stages did not show a leaf infection in barley accessions.

While the leaf infection for the oat controls decrease after tillering (24d) was complete, the leaf infection in barley accessions already decreased after seedling growth (10d) (Figure 12A). Most notably, the leaf infection of VxS 152 at seedling growth (10d) is one of the highest but no longer shows leaf infection during tillering (24d), during tillering however, the leaves of VxS 152 started to show unidentified brown spots (Figure 12B). The brown spots developed on all barley accessions in 37-day old plants and older plants, except for L94 and oat controls (Figure 12B). VxS 152 already showed signs of the brown spots during seedling growth (10d). In plants older than 10 days VxS 152 no longer exhibited teliospore development (Figure 12). Further, SusPtrit showed first signs of the brown spots during the tillering stage (24d) while also still developing pustules. During stem extension (37d) neither pustules nor teliospores were observed for VxS 152 and SusPtrit but also SusPmur, and Trigo Bisasa were uninfected (Figure 12). However all four barley accessions were heavily infected by the unidentified brown spots. It should also be noted that during stem extension, some plants with the brown spots exhibited newly developing leaves without brown spots. However, these new leaves did not show signs of *Pga* leaf infection because they most probably developed after inoculation.

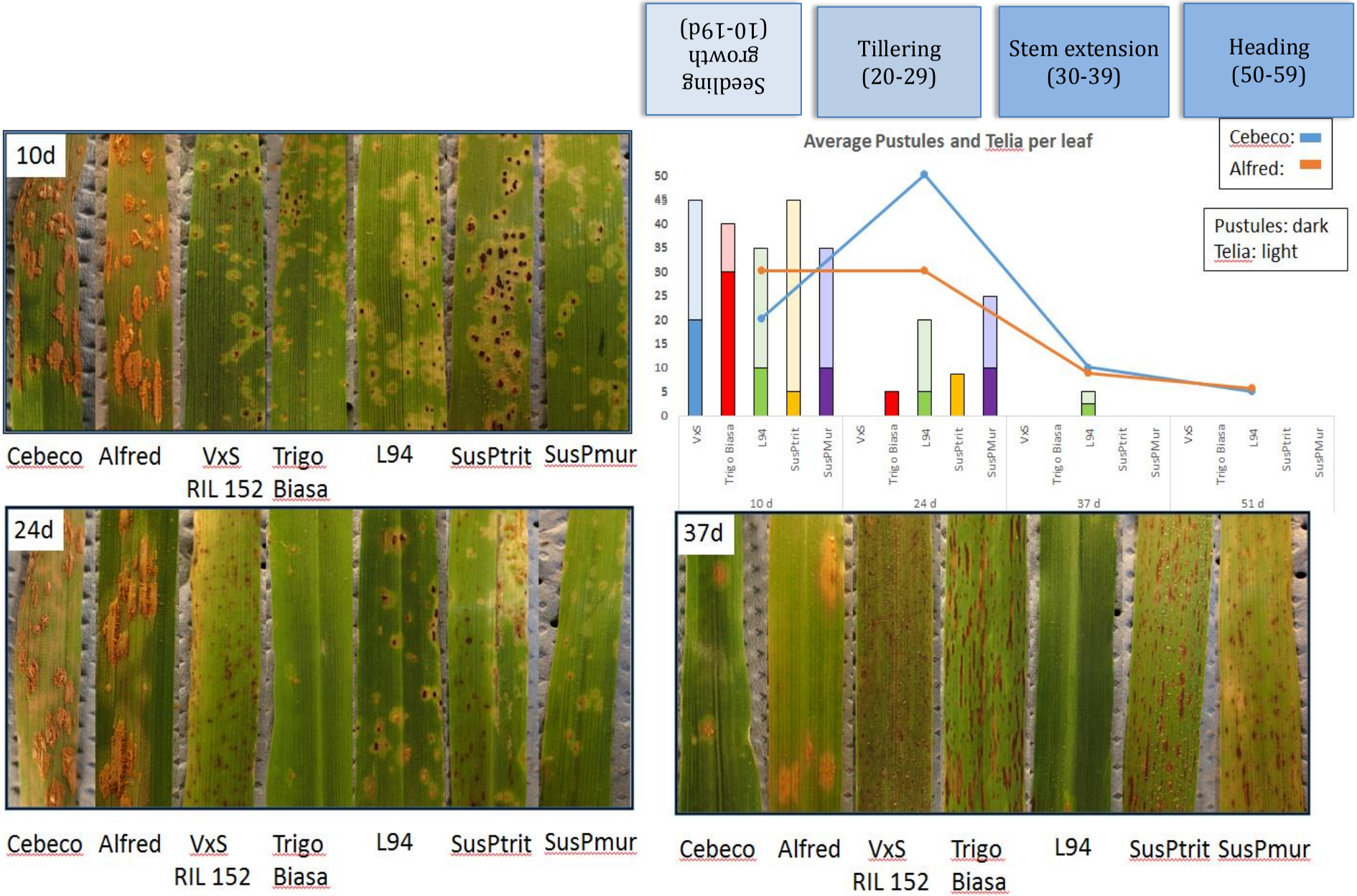
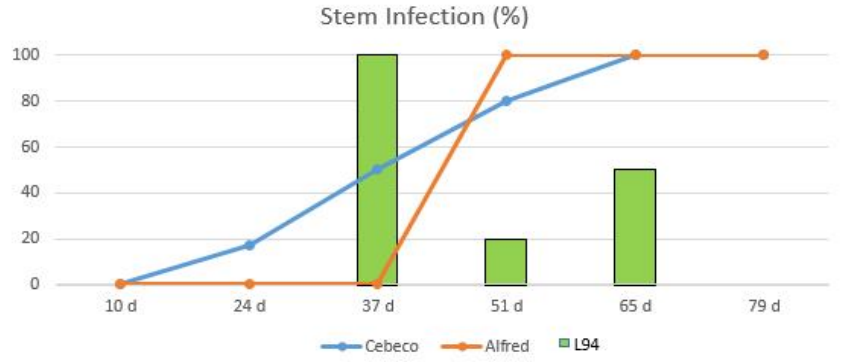


Figure 12: A: Stacked bars with pustules (dark) and teliospores (light) indicating barley infection and Lines indicate oat infection with pustules at four different developmental stages (10d, 24d, 37d, and 51d with Puccinia graminis f. sp. Avenae isolate Ingeberga 12 dai. **B:** Infection of oat (Cebeco and Alfred) and barley (VxS 152, Trigo Biasa, L94, SusPtrit, and SusPmur) leaves. Presented plants were 10 d, 24 d and 37 d old at time of inculation. Stage conversion to days according to Zadoks scale..

During stem extension and heading (37d and 51d), the leaf infection of the oat control decreased considerably and the size of the pustules decreased compared to earlier developmental stages (Figure 12). At the same time the pustules on the stems first became visible in oat controls (Figure 13). Similarly, during stem extension (37d) L94 has the lowest infection frequency compare to earlier development stages, while simultaneously the stem infection is highest during this development stage (Figure 13). While the leaf infection was most pronounced during early development, the later developmental stages saw an increase in the stem rust infection frequency. While Cebeco oat showed continuously increasing stem infection frequencies, the stem infection of Alfred exploded from 0% on 37-day old plants to 100% on 51-day old plants (Figure 14A). With increasing developmental stages oat stem infection amount also steadily increases and plateaus for 65- and 79-day old plants (Figure 13B). Barley stem infection was only observed on L94, all other barley accessions were not infected (Figure 13B). Clearly, L94 stem infection also exploded from 0% to 100%, but earlier than oat and subsequently decreased below 100% infection frequency for more advanced developmental stages (Figure 13B). Not only does L94 show a higher infection frequency than oat on 37-day old plant but also showed a higher amount of infection (Figure 13). The infection amount and the frequency decrease in more advanced developmental stages (Figure 13B).

A



B

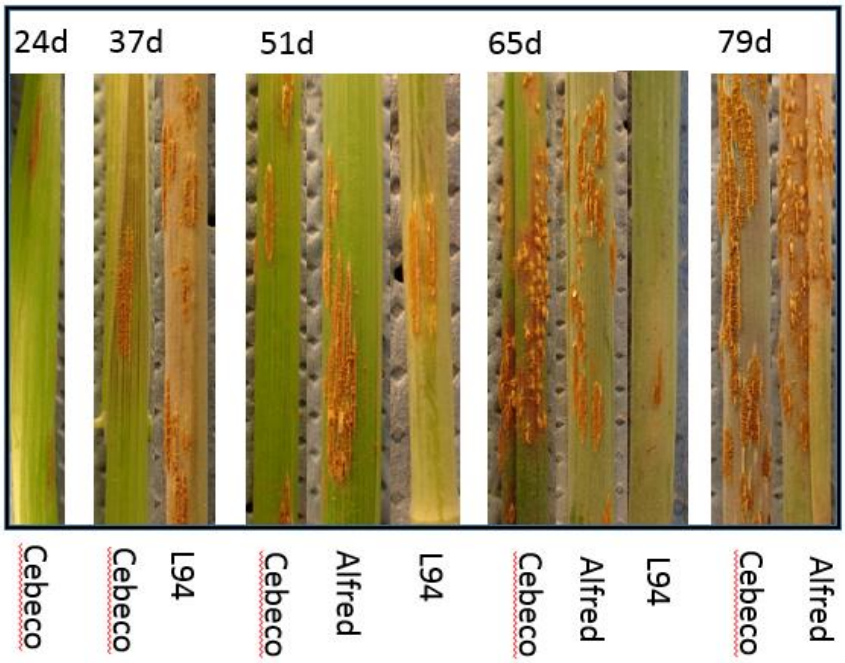


Figure 13 A: Bars indicating stem infection of *Puccinia graminis* f. sp. *avenae* isolate Ingeberga 12 dai on barley accession L94 compared to Lines showing Cebeco and Alfred Oat stem infection frequency (%)at five different developmental stages (24d, 37d, 51d, 65d, and 79d) with **B:** Visual stem infection of five developmental stages.

3. Discussion

3.1. Fine Mapping *Rpgaq1*

For *Rpgaq1*, it was possible to reduce the target region from ~50 cM to a 1 cM interval between marker J1 and J3 (Figure 4). There are ten heterozygous recombinants available for this interval, which can be used for future fine mapping experiments (Table 13). Also, new SNPs should be developed in the 0.9 cM interval between J1 and J2 for better saturation of the target region.

We were surprised by the dominance rate observed in the different generations. In the F_2 generation, we concluded on the additive gene action based on the observations of an intermediate average VIS approximately 50% higher than the homozygous resistant genotype group and approximately 50% lower than the homozygous susceptible genotype group (Figure 2B). Contrarily, F_4 generations from the third seedling test revealed heterozygous groups that were all as resistant as the resistant homozygous genotype groups, implicating a dominant gene action (Figure 8). Dominance for *Rpgaq1* was also reported in the first and second seedling test, however the unreliable phenotype has limited credibility. An explanation for the differing dominance rates between generations could be the segregation of one or more unknown minor genes or QTLs in the background of the *Rpgaq1* F_2 mapping population. Especially, considering that successive generations no longer showed intermediate VIS in the heterozygous genotype groups, it is possible that the loci of minor genes or QTLs were homogenized through continued selfing. Additionally, homozygous susceptible genotype groups of the third seedling test (F_4), varied for the degree of susceptibility between the tested families and their respective origins (Figure 8). Those families that related to the F_2 individual 2.54 showed the highest overall susceptibility, also compared to the susceptible control (Figure 8). The segregation of an additional gene in the F_2 generation could explain the difference in susceptibility between the F_3 recombinant families. Because, if the *Rpgaq1* mapping population segregated for a minor QTL, which is homozygous resistant in the susceptible control (RIL 152), than RIL 152 could be incompletely susceptible. Hence, there is a possibility of observing significantly more susceptible individuals in segregating families compared to the susceptible control (Figure 8).

The prospect of an additional gene led us to consider the possibility that the reported LOD profile for *Rpgaq1* contains an additional QTL at the second peak around 80cM reported by Dractos et al. (2016) (Figure 1). This would be plausible considering that F_2 individual 2.54 is homozygous SusPtrit for M11 which maps to 80cM on the VxS SNP map, coinciding with the location of the second peak (Appendix 1). Using a linkage map constructed with AFLP and SSR markers by Jafary et al. (2006) to genotype all VxS RILs it was possible to examine the parental genotype of the fine mapping cross (RIL 152 x RIL 110). However, the parental linkage map revealed missing genotype data around 80cM (Table 19). Yet, to explain that RIL 152 is incompletely resistant because it is homozygous resistant Vada for the locus around the LOD peak, and RIL 110 is homozygous susceptible SusPtrit, it would require a rare double crossover between E37M50-103 and E38M55-433 in RIL 110 (Table 19). Because this is a rare event, we chose to evaluate the parental genotypes of all other QTLs identified to be effective against *Pga* by Dractos et al. (2016) (Table 20).

		Confirmed Genotype		Assumed Genotype	
AFLP Marker	Consensus Map position (cM)	RIL 110	RIL 152	2.54 Families	2.14 Families
E37M50-103	72,60	VV	SS	SS	VV
E42M54-424	79,63	Unknown	Unknown	SS	VV
E38M55-433	83,86	VV	VV	VV	VV

Table 19: Genotype based on AFLP markers for 1H mapping population parents RIL 110 and RIL 152. VV=homozygous Vada; SS=homozygous SusPtrit, Unknown= unknown genotype. Developed by Jafary et al. 2006.

All resistance QTLs identified by Dracatos et al. (2016) segregating in the *Rpgaq1* mapping population are homozygous SusPtrit for RIL 152 except *Rpgaq3* on the 6H chromosome (Table 20). RIL 152 is homozygous Vada and RIL 110 is homozygous SusPtit, resulting in the segregation of *Rpgaq3* in the F_2 generation. However, *Rpgaq3* was only effective against *Pga* isolates Ingeberga and Pattala and not effective against *Pga* Evertsholm (Dracatos et al. 2016). But it is common that minor QTLs are just below the threshold of detection and often need independent studies to confirm their existence (Acquaah, 2012). This should be especially relevant for isolates with a low virulence compared to other isolates, such as the case reported by Dracatos et al. 2016 for *Pga* Evertsholm compared to *Pga* Ingeberga and *Pga* Patalla.

			1H Map Pop			
LG	QTL	Position	RIL 110	RIL 152	Resistance allele donor	Isolate specificity
1H	*Rpgaq1*	53,03	VV	SS	Vada	I, P, E
2H	*Rpgaq2*	86,713	VV	SS	Vada	I, P
6H	*Rpgaq3*	54,03	SS	VV	Vada	I, P
7H	*Rpgaq5*	5,97	VV	VV	SusPtrit	P,E
7H	*Rpgaq4*	118,024	VV	SS	Vada	I, P

Table 20: parental genotypes of 5 QTLs identified in VxS cross to affect Pga resistance for 1H mapping population (MP) and 7H mapping population. VV=homozygous Vada, Homozygous SusPtrit. I: *Pga* Ingeberga P: *Pga* Pattala E: *Pga* Evertsholm

Hence, an important issue that should be considered is the statistical reliability of the sample size for interval mapping (Doerge 2002). In this case, only 35 out of 152 susceptible lines in the VxS mapping population were used for the QTL mapping of *Pga* Evertsholm. Therefore, it is questionable if the QTL mapping was powerful enough to detect smaller effect QTLs. In case of *Rpgaq3*, the LOD profile does not show any sign of a peak below the LOD threshold (Nansamba unpublished). However, *Rpgaq3* is the only mapped QTL affecting *Pga*, that fits the hypothesis which believes RIL 152 to carry resistance allele for a minor QTL. Alternatively, an unmapped QTL could also condition the phenotypic difference between homozygous susceptible genotype groups of selected recombinant families and the susceptible control. However, *Rpgaq3* should be considered because if the QTL has a non-specific function this could explain the phenotypic difference observed between 2.14 and 2.54 families.

Both *Pga* Ingeberga and *Pga* Pattala have been implicated to be affected by *Rpgaq3* (Table 20). Previously, Jafary et al. (2006) mapped a QTL (*Rphq3)* effective against heterologous rusts to the same chromosome interval as *Rpgaq3*, between 50 and 65 cM on the 6H chromosome in the VxS consensus map. Jafary et al. (2006) demonstrated that *Rphq3* is isolate non-specific and effective to 5 out of 8 heterologous rust species tested. If *Rphq3* is the same QTL as *Rpgaq3* described by Dracatos et al. (2016), additional tests should be conducted to confirm the isolate specificity of *Rpgaq3*. This is very important because previous studies have suggested that in some cases QTLs can appear isolate non-specific but instead this region contains a gene cluster with different genes each with a more narrow spectrum (Niks, 2014).

3.2. Fine Mapping *Rpgaq5*

Compared to the LOD profile of *Rpgaq1*, the LOD profile for *Rpgaq5* was much narrower, starting with 3 cM which was later reduced to a 2 cM interval between M15 and J6 (Table 15). The observed gene action was dominant in all cases. For the 2 cM target region there are 11 heterozygous recombinants available as well as 5 homozygous recombinants (Table 18). They should be used as resources for further fine mapping. Additionally, new SNPs should be developed to further elucidate the target region. Especially the 1.6 cM region between M17 and J6 needs to be saturated with newly developed SNPs.

We noticed unexpected infection differences between the homozygous susceptible genotype group (Vada) and the susceptible control (RIL 152) (Figure 11). In the *Rpgaq5* mapping population, RIL 152 was fully susceptible and the homozygous Vada groups were significantly less susceptible than RIL 152, except family 2.31.27 which only showed tendencies to be significantly less susceptible ($p<0.1$) (Table 16). Additionally we observed infection differences between the five family's homozygous Vada genotype groups (Figure 15). The phenotypic differences within the genotype group could be explained by a segregating gene or QTL in the background of the *Rpgaq5* mapping population. Moreover, the homozygous Vada group from the F_2 population, along with the five homozygous Vada genotype groups from the third seedling test, demonstrated three distinct infection levels with an approximate 50% infection drop between each infection level (Figure 15). The infection levels are as follows: 1) the lowest infection: F_3 family 2.69 and F_4 family 2.4.48, 2) the intermediate infection: the F_2 mapping population and F_4 family 2.4.43 and 3) the highest infection: F_4 families 2.40.30 and 2.31.27 (Figure 15).

The three infection levels could be explained by the presence of a segregating minor QTL which is homozygous resistant in the low infection families, segregating in the intermediate families, and homozygous susceptible in the high infection families (Figure 15). The segregation of an unknown QTL in the homozygous vada genotype groups of the 2.4.43 family and the F_2 population would explain the intermediate score because, in the F_4 generation the coefficient of inbreeding suggests 12.5% expected heterozygosity, 43.75 % homozygous susceptible and 43.75% homozygous resistant genotypes. Adding up the high (43.75%) and low (43.75%) phenotypes, conditioned by the genotypes, would therefore produce a more or less intermediate score compared to the other infection levels. Also, we assume that high infection families 2.40.30 and 2.31.27 and RIL 152 are homozygous susceptible, and that the significantly lower infection frequency between the tested families and RIL 152 is conditioned by a generally lower infection of all *Rpgaq5* individuals. Because, compared to susceptible *Rpgaq1* individuals, the susceptible *Rpgaq5* individuals were always less susceptible. For instance, the difference between homozygous susceptible genotype groups of *Rpgaq5* and *Rpgaq1* in the F_2 populations is a 37% lower VIS.

Generally, *Rpgaq1* families are all either equally susceptible or more susceptible compared to the susceptible control. Yet the third seedling test for *Rpgaq5* showed that the RIL 152 is roughly 30% more susceptible compared to 2.40.30 and 2.31.27 which is close to the 37% infection difference mentioned above (Appendix 1 and Figure 15). Hence, we hypothesize that the infection difference between the susceptible control and 2.40.30 and 2.31.27 was not conditioned by different allele compositions in the segregating QTL but by infection differences of a different origin. Therefore, we will consider the susceptible control equally susceptible compared to families 2.40.30 and 2.31.27 for the sake of argument in the following section.

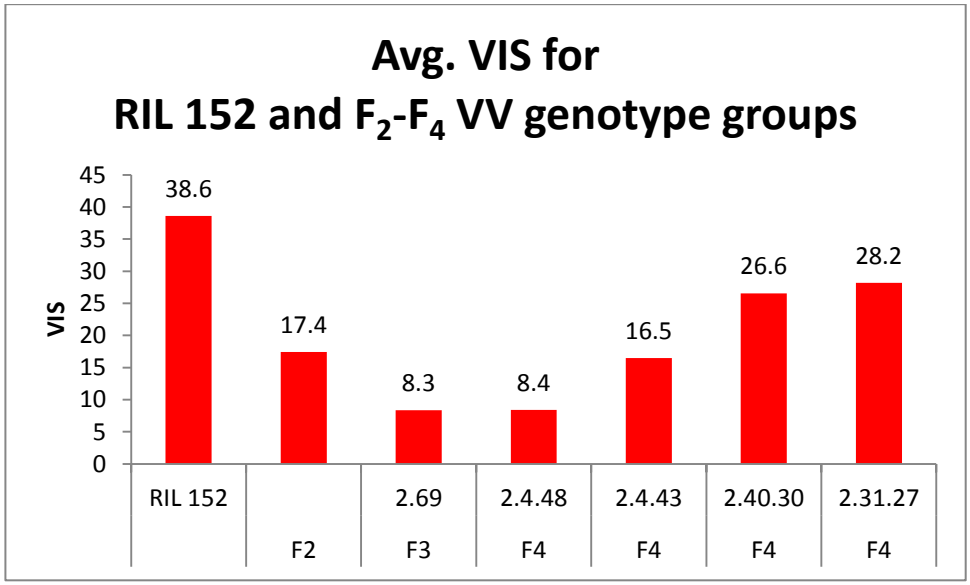


Figure 15: The average visual infection sites (VIS) for the positive contol, F_2 idividuals, F_3 familiy 2.69 and F_4 familiy 2.4.48, 2.4.43, 2.40.30, 2.31.27 genotyped for homozygous Vada *Rpgaq5*

Understanding the infection difference between the VV genotype groups is a priority and as hypothesized extensively above might be conditioned by other QTLs segregating in the background. Therefore, we checked the parental genotype for loci which had previously been indicated as harbouring resistance QTLs effective against *Pga* (Dracatos et al. 2016). But, contrary to *Rpgaq1* we expect that RIL 152 is homozygous susceptible for all QTLs because it is never less susceptible than the family genotype groups (Figure 11). This only leaves *Rpgaq2* on the 2H chromosome as a potential candidate because it is the only segregating locus which is homozygous SusPrit in RIL 152 (Table 21).

LG	Position	QTL	RIL143	RIL152	Resistance allele donor	Affective Isolates
1H	53.03	*Rpgaq1*	SS	SS	Vada	I, P, E
2H	86.71	*Rpgaq2*	VV	SS	Vada	I, P
6H	54.03	*Rpgaq3*	SS	VV	Vada	I, P
7H	5.97	*Rpgaq5*	SS	VV	SusPtrit	P, E
7H	118.02	*Rpgaq5*	SS	SS	Vada	I, P

Table 21: parental genotypes of 5 QTLs identified in VxS cross to affect *Pga* resistance for *Rpgaq5*. VV=homozygous Vada, Homozygous SusPtrit. I: *Pga* Ingeberga P: *Pga* Pattala E: *Pga* Evertsholm

Similar to several other QTLs, *Rpgaq2* only showed effectiveness against *Pga* isolates Pattala and Ingeberga. However, *Rpgaq2* also showed effectiveness against *Pga* Pattala and Ingeberga in two mapping populations: 1) VxS and 2) Cebeca Caba x SusPtrit (Dracatos et al. 2016). In any case, we are not confident that 35 susceptible RIL lines are sufficient to pick up small QTL effects during the QTL analysis conducted by Nansamba (unpublished). Hence, the involvement of *Rpgaq2* in the resistance reaction against *Pga* Evertsholm needs to be confirmed in future experiments. One possibility is to inoculate RILs with higher amounts of rust, producing more phenotypically susceptible lines used for QTL detection. Provided more genetically susceptible lines exist. This may identify minor QTLs effective to *Pga* Evertsolm beyond *Rpgaq2*. Mackay et al. (2009) suggested that the number of individuals needed to map QTLs increases as the average phenotype difference between genotype groups decreases.

Additionally, *Rpgaq2* appears to map to the same chromosome region (80-100 cM) of the VxS consensus map as a QTL effective to only one out of three tested *Puccinia triticina* isolates reported by Jafary et al. (2008). The QTL was detected with a relatively low LOD of 3.9 (Jafary et al, 2006). This is similar to the LOD of *Rpgaq2:* 3.64 and 4.45 for *Pga* Ingeberga and *Pga* Pattala respectively. As mentioned before, *Pga* Evertsholm had a lower infection frequency than *Pga* Ingeberga and *Pga* Pattala on SusPtrit. Therefore, this could explain why the LOD peak for *Rpgaq2* was not above the significance threshold (LOD=~ 2) (Nansamba unpublished). If more heterologous rusts can identify *Rpgaq2* as a resistance QTL, it is doubtful if *Rpgaq2* is isolate specific as stated by Dracatos et al. (2016). Especially, considering the QTL maps to more than one mapping population. More disease tests are needed to clarify the role of *Rpgaq2* in the resistance reaction against *Pga* Evertsholm. We strongly advise to use higher inoculum densities for a better QTL detection.

3.3. Adult Plant Test

To confirm the observation by Martens et al. (1977) that barley adult plants can be infected by *Pga*, we tested different developmental stages of five barley accessions for their susceptibility to *Pga.* While the susceptibility of the barley accession decreased with increasing developmental stages we observed that one accession (L94) showed leaf infections past the seedling stage and the tillering stage. Thus, we concluded that the observations by Marteens et al. (1977) are plausible for susceptible adult plants.

However, due to the occurrence of the unidentified disease on all other barley accessions it was not possible to conclude if L94 was the only accession susceptible to *Pga* Ingeberga or if a contaminating effect of the brown spots supressed the leaf infection of *Pga* Ingeberga in all other barley accessions. We assume that the unidentified brown spots were cause by ramularia leaf spot, which leads to extensive and premature leaf death (Oxley et al. 2012). Keeping plants free from physiological stress is essential to preventing ramularia leaf spot (Oxley et al. 2012). Nevertheless, early development stages that do not show brown spots, demonstrated that the level of leaf infection varied between the barley accessions and that some accessions were prone to the development of teliospores while others were not (Figure 12). The haustorium penetration success could be further tested with some transient experiments using GFP, green fluorescent protein, and microscopy. Overall, L94 was susceptible to *Pga* Ingerbera however at a lower leaf infection level than oat varieties (Figure 12).

During later development stages, we observed an increase in stem infections in both oat control plants and the only susceptible barley accession L94 (Figure 13). For both oat controls we observed an increasing infection amount in 37 day old plants and older, while L94's infection amount decreased with increasing developmental stages (Figure 13B) and the 100% stem infection was also not maintained past 37day old plants (Figure 13A). Therefore, it is possible to state that while the infection of adult barley plants by heterologous rust *Pga* is possible but that the infection is most pronounced at the seedling stage.

3.4. Outlook and Recommendations

In summary, the presented study has generally not been successful in fine mapping the QTLs far enough to start the process of screening BAC libraries to establish a physically map of *Rpgaq1* and *Rpgaq5*.Continued fine mapping could be achieved by testing the heterozygous and homozygous recombinants identified for either QTL. However, more SNPs need to be develop to saturate marker intervals and fine map both QTLs to such an extend to allow physical mapping. Also, as the presented report shows, it is not entirely clear if additional genes or QTLs are segregating in the background of each mapping population. Hence, confirming the results of the interval mapping presented by Dracatos et al. 2016 should bring clarity on the isolate specificity of the QTLs in response to a *Pga* infection. As mentioned in previous sections, the inoculation dose of *Pga* Evertsholm should be increased to determine if this results in more than 35 susceptible RILs out of 152 RILs, which could improve the power of statistically predicting the locations of QTLs affecting the infection with Pga Evertsholm. Additionally, it should be confirmed if the references of previously identified QTLs co-locate with the studied QTLs. Among others, this should provide some insight on the isolate specificity of the studied QTLs. Because, maybe the resistance of SusPtrit to *Pga* Evertsholm and the susceptibility to *Pga* Ingerberga and *Pga* Pattala is conditioned by quantitative isolate specificity. Finally, the adult plant test confirmed that adult plants can also be infected by heterologous pathogens. However, it was not possible to see if quantitative variation exists for the degree of infection. This should be confirmed with histological studies.

4. Acknowledgement

I wish to extend sincere gratitude to my thesis supervisor, Rients Niks for his continuous guidance, timely feedback, patience and advice that has taught me so much. I also acknowledge Peter Michael Dracatos for his practical tips and remote supervision. I would also like to thank Anton Vels for the technical support and Mathilde Deniau and Yajun Wang for the guidance rendered during laboratory experiments.

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6. Appendix

6.1. Appendix 1: *Rpgaq1* mapping population F_2 Genotype

A

		M1	M2	M4	M6	M7	M8	M9	M10	M11	M12	M13
	consensis map	41,9	42,428	51,7	58,7	61,16	65,32	67,72	74,78	79,11	89,9	103,27
	VxS consensus	49,195	54,384	55,582	70,748	72,515	88,144	88,733	95,906	99,773	109,303	117,954
Plant no.	**VIS**											
2-1	0	v	v	v	v	v	h	h	h	h	unknown	s
2-2	41	h	h	h	h	h	s	h	s	s	h	v
2-3	0	h	h	h	h	v	h	h	h	h	v	Negative
2-4	5	h	h	h	h	h	Unknown	Unknown	s	Negative	h	Negative
2-6	3	s	unknown	h	h	s	Unknown	v	v	Unknown	h	h
2-8	0	s	h	h	h	h	h	h	h	h	s	s
2-9	2	h	h	h	h	h	v	Negative	s	s	s	Negative
2-10	0	h	h	h	h	v	h	h	h	h	s	v
2-12	**1**	**s**	**s**	**h**	**h**	**h**	**Unknown**	**h**	**h**	**h**	**v**	**v**
2-14	18	h	h	h	h	h	h	h	h	h	h	v
2-15	3	h	h	h	h	h	v	s	s	s	s	Negative
2-16	40	h	h	h	h	h	h	Negative	h	s	**h**	**v**
2-17	21	h	h	h	h	v	Unknown	Unknown	Negative	s	h	Unknown
2-18	15	h	h	h	h	h	h	h	h	h	v	v
2-20	12	h	h	h	h	h	h	Negative	h	h	v	s
2-21	24	h	h	h	h	h	h	h	h	h	v	v
2-25	0	h	h	h	h	h	h	h	Negative	Negative	h	v
2-26	25	h	h	h	h	h	h	h	h	Unknown	v	v
2-27	5	h	h	h	h	h	h	h	h	h	h	h
2-28	7	h	h	h	h	h	h	h	h	h	h	h
2-29	0	h	h	h	h	v	v	v	v	v	v	v
2-31	4	h	h	h	h	h	h	h	h	Unknown	h	v
2-34	1	h	h	h	h	h	h	h	v	Unknown	h	h
2-37	0	h	h	h	h	h	h	s	v	v	h	Unknown
2-38	**1**	**h**	**h**	**h**	**h**	**v**	**v**	**v**	**v**	**v**	h	s
2-40	2	v	h	h	h	h	h	Negative	s	s	v	v
2-41	4	v	h	h	h	h	h	h	Negative	h	h	h
2-43	0	h	h	h	h	h	s	h	h	h	v	Negative
2-45	26	h	h	h	h	v	v	v	v	v	v	h
2-49	15	v	v	h	h	h	h	h	h	h	h	h
2-52	6	h	h	h	h	h	h	h	v	h	h	s
2-53	9	h	h	h	h	h	h	h	v	v	v	s
2-54	24	h	h	h	h	h	h	h	h	s	v	h
2-56	23	h	h	h	h	h	h	h	h	h	h	h
2-58	21	h	h	h	h	h	h	h	h	h	s	s
2-59	2	h	h	h	h	h	h	h	s	s	v	v
2-60	8	h	h	h	h	h	h	h	h	h	s	Negative
2-67	1	h	h	h	h	h	s	h	h	v	v	v
2-68	1	h	h	h	h	v	v	v	v	v	h	h
2-69	14	h	h	h	h	h	s	s	s	s	v	s
2-70	10	h	h	h	h	h	Negative	v	v	v	v	s
2-5	14	h	s	s	s	s	s	s	s	s	s	s
2-7	10	h	s	s	s	s	s	s	s	s	s	s
2-11	7	s	s	s	s	s	s	s	Negative	h	h	h
2-13	13	s	s	s	s	s	s	s	s	s	v	Negative
2-19	5	s	s	s	s	s	s	s	Unknown	s	v	h
2-22	16	s	s	s	h	h	h	h	h	h	s	s
2-30	38	s	s	s	s	h	h	h	h	h	v	v
2-35	18	s	s	s	s	s	s	s	s	s	h	h
2-36	22	s	s	s	s	h	h	h	v	v	s	s
2-42	59	s	s	s	s	s	s	s	v	s	v	v
2-47	24	s	s	s	s	s	s	s	s	s	h	h
2-50	13	s	s	s	s	s	s	v	s	Unknown	v	v
2-62	48	s	s	s	s	s	s	s	s	s	s	s
2-39	12	h	h	unknown	v	v	v	v	h	s	h	h
2-44	4	h	h	unknown	v	v	v	v	h	v	h	h
2-63	18	v	v	unknown	h	h	h	h	v	v	v	h
2-23	0	v	v	v	h	h	h	h	h	h	h	h
2-24	0	v	v	v	v	v	Unknown	Unknown	Negative	h	h	h
2-32	1	h	v	v	v	v	v	v	Negative	s	h	h
2-33	2	v	v	v	v	v	v	v	v	Unknown	**h**	**s**
2-46	7	v	v	v	v	v	v	v	v	v	v	v
2-48	0	v	v	v	v	v	v	v	v	Unknown	v	v
2-51	1	v	v	v	v	v	Negative	v	v	v	v	v
2-55	0	v	v	v	v	v	v	v	v	v	h	h
2-57	1	v	v	v	v	v	v	v	v	v	v	s
2-61	**0**	**v**	**v**	**v**	**h**	**h**	**h**	**h**	**h**	**h**	h	s
2-64	0	v	v	v	v	v	v	v	v	v	v	v
2-65	1	v	v	v	v	v	v	v	v	Negative	s	s
2-66	0	v	v	v	v	v	h	h	h	h	v	v
total	10,4											
ss	22,1											

B

mean VIS	v	11,9	10,6	12,1	12,5	11,1	9,9	13,1	13,4	9,7	11,0	12,7
	h	9,1	9,8	10,4	9,4	9,8	10,3	9,9	9,0	8,1	9,1	9,4
	s	12,1	12,5	10,4	10,9	11,4	11,7	9,4	11,9	12,0	12,1	10,4
Expected	v	17,5	17,25	16,25	17,5	16,75	14,25	15,5	15	14,75	17,5	15,25
	h	35	34,5	32,5	35	33,5	28,5	31	30	29,5	35	30,5
	s	17,5	17,25	16,25	17,5	16,75	14,25	15,5	15	14,75	17,5	15,25
Observed	v	17	15	14	14	21	17	23	28	18	28	29
	h	39	40	38	43	36	32	33	28	25	29	21
	s	14	15	13	12	10	11	13	11	21	12	20
Chi Sq p<0.01		0,557	0,481	0,388	0,119	0,136	0,427	0,125	0,002	0,132	0,011	0,000

Figure Appendix 1: Genotyping barley 1H mapping population (RIL 152 x 110) using 11 SNPs. Blue s= SusPtrit; grey h= heterozygous; red v= Vada. Visual infection sites 12 dai with *Puccinia graminis* f. sp. avenae isolate Evertsholm. **B:** Mean VIS according to allele and SNP. Chi Square test: red= p<0.01; orange= p<0.1)

6.2. Appendix 2: *Rpgaq5* mapping population F_2 Genotype

A

		M15	M17	M21	M22	M23	M19
location consensus map		1,818	3,46	6,174	7,839	8,055	5,886
location VxS		6,024	6,870	10,626	11,215	11,804	10,612
Sample	**VIS**						
2_1	2	HH	HH	HH	HH	HH	HH
2_4	0	h	HH	HH	HH	HH	HH
2_5	0	HH	HH	HH	HH	HH	HH
2_11	0	HH	HH	HH	HH	HH	HH
2_12	1	HH	HH	HH	Negative	HH	VV
2_15	0	HH	HH	HH	HH	HH	Unknown
2_19	1	HH	HH	HH	HH	HH	HH
2_25	1	HH	HH	HH	HH	HH	HH
2_27	3	HH	HH	HH	HH	HH	HH
2_28	1	HH	HH	HH	HH	HH	HH
2_31	1	HH	HH	HH	HH	HH	Unknown
2_34	1	HH	HH	HH	HH	HH	HH
2_40	1	HH	HH	HH	HH	HH	HH
2_42	0	HH	HH	HH	HH	HH	HH
2_46	0	HH	HH	HH	HH	HH	HH
2_48	0	HH	HH	HH	HH	HH	HH
2_49	2	HH	HH	HH	HH	HH	HH
2_50	0	HH	HH	HH	HH	HH	HH
2_51	0	HH	HH	HH	HH	HH	HH
2_53	0	HH	HH	VV	VV	VV	VV
2_54	2	HH	HH	HH	HH	HH	HH
2_55	1	HH	HH	HH	Negative	HH	HH
2_57	2	HH	HH	HH	HH	HH	HH
2_61	2	HH	HH	HH	HH	HH	HH
2_65	0	HH	HH	HH	HH	HH	HH
2_66	0	HH	HH	HH	HH	HH	HH
2_69	1	VV	HH	HH	Negative	HH	HH
2_70	1	HH	HH	SS	SS	SS	VV
2_24	0	HH	negatiVVe	Negative	Negative	HH	HH
2_64	1	HH	negatiVVe	Negative	Negative	HH	Negative
2_3	1	HH	negatiVVe	Unknown	HH	HH	HH
2_7	0	HH	negatiVVe	HH	HH	HH	Negative
2_9	3	HH	negatiVVe	Negative	Negative	HH	Unknown
2_18	1	SS	SS	SS	SS	SS	SS
2_20	0	SS	SS	SS	SS	SS	SS
2_23	1	SS	SS	SS	SS	SS	SS
2_30	0	SS	SS	SS	SS	SS	VV
2_33	0	SS	SS	SS	Unknown	SS	VV
2_35	1	SS	SS	SS	SS	SS	SS
2_39	0	SS	SS	SS	SS	SS	SS
2_43	1	SS	SS	Unknown	SS	SS	Negative
2_47	0	SS	SS	SS	SS	SS	SS
2_58	2	SS	SS	SS	SS	SS	Negative
2_59	3	SS	SS	SS	SS	SS	SS
2_62	1	SS	SS	SS	SS	SS	SS
2_68	2	SS	SS	SS	SS	SS	VV
2_16	**1**	VV	unknown	VV	**VV**	**VV**	**VV**
2_37	7	VV	unknown	VV	VV	VV	VV
2_2	25	VV	VV	VV	VV	VV	VV
2_6	17	VV	VV	VV	VV	VV	VV
2_8	20	VV	VV	VV	VV	VV	VV
2_10	58	VV	VV	VV	VV	VV	VV
2_13	8	VV	VV	VV	VV	VV	VV
2_14	32	VV	VV	VV	VV	VV	VV
2_17	28	VV	VV	Unknown	VV	VV	VV
2_21	9	VV	VV	VV	VV	VV	VV
2_22	22	VV	VV	VV	VV	VV	VV
2_26	2	VV	VV	VV	VV	VV	VV
2_29	19	VV	VV	VV	VV	VV	VV
2_32	14	VV	VV	VV	VV	VV	VV
2_36	1	VV	VV	VV	Negative	VV	VV
2_38	15	VV	VV	VV	VV	VV	VV
2_44	2	VV	VV	VV	VV	VV	Unknown
2_45	23	VV	VV	VV	Unknown	VV	VV
2_52	9	VV	VV	VV	Negative	VV	VV
2_56	0	VV	VV	VV	VV	VV	VV
2_60	54	VV	VV	VV	VV	VV	VV
2_63	39	VV	VV	VV	Unknown	VV	Unknown
2_67	1	VV	VV	VV	VV	VV	VV
2_41	12	VV	VV	VV	VV	VV	VV

B

mean VIS	v	6,1	7,2	6,5	4,6	6,4	7,5
	h	6,7	7,1	6,4	5,8	6,5	6,9
	s	6,5	6,5	7,3	7,2	6,9	4,8
Observed	v	25	22	24	21	25	28
	h	32	27	27	25	31	25
	s	14	14	13	13	14	8
	total	70	63	64	59	70	61
Expected	v	17,5	15,75	16	14,75	17,5	15,25
	h	35	31,5	32	29,5	35	30,5
	s	17,5	15,75	16	14,75	17,5	15,25
Chi Squ		0,124	0,190	0,069	0,170	0,112	0,001

Figure Appendix 2A: Genotyping barley 7H mapping population (RIL 143 x 152) using 6 SNPs. Blue s= SusPtrit; grey h= heterozygous; red v= Vada. Visual infection sites 12 dai with *Puccinia graminis* f. sp. avenae isolate Evertsholm. **B:** Mean VIS according to allele and SNP. Chi Square test: red= p<0.01)

6.3. Appendix 3: F_2 Linkage Map Distances

6.3.1. *Rpgaq1* linkage map based on F_2 genotype

Marker pair	M1-M2	M2-M4	M4-M6	M6-M7	M7-M8	M8-M9	M9-M11
Marker IDs (last 3 digits)	276 -548	548-177	177-643	643-562	562-198	198-506	506-611
Recombinations	7	1	3	10	10	8	14
Gametes	138	128	130	134	112	104	104
RF	0.05	0.01	0.02	0.07	0.09	0.08	0.13
RF Distance	5 cM	1 cM	2 cM	7 cM	9 cM	8 cM	13 cM

Table Appendix 3A: Marker distances according to recombination frequencies (RF) of F_2 mapping population for 1H barley chromosome

6.3.2. *Rpgaq5* linkage map based on F_2 genotype

Marker pair	M15-M17	M17-M21	M21-M22	M22-M23
Marker IDs (last 3 digits)	028-095	095-615	615-959	959-792
Recombinations	1	2	0	0
Gametes	126	122	112	129
RF	0.01	0.02	0	0
RF Distance	1 cM	2 cM	0 cM	0 cM

Table Appendix 3B: Marker distances according to recombination frequencies (RF) of F_2 mapping population for 7H barley chromosome

6.4. Appendix 4: Recombinant Screening Linkage Map Distances

6.4.1. *Rpgaq1* linkage map based on recombination screening

Marker pair	M2-M4	M4-J1	J1-J2	J2-J3	J3-J4	J4-J5	J5-M6
SNP IDs (last 3 digits)	578-177	177-869	869-483	483-637	637-264	264-042	042-643
Recombinations	13	23	9	1	1	3	4
Gametes	966	954	958	950	950	950	950
RF	0.013	0.024	0.009	0.001	0.001	0.003	0.004
RF Distance	1.3 cM	2.4 cM	0.9 cM	0.1 cM	0.1 cM	0.3 cM	0.4 cM

Table Appendix 4A: **:** Marker distances according to recombination frequencies (RF) of F_3 mapping population for barley 1H chromosome following recombinant screening

6.4.2.*Rpgaq5* linkage map based on recombination screening

Marker pair	M15-M17	M17-J6	J6-J7	J7-J8	J8-M21
SNP IDs (last 3 digits)	028-095	095-557	557-631	631-396	396-615
Recombinations	3	12	8	2	17
Gametes	736	754	746	740	750
RF	0.004	0.016	0.011	0.003	0.023
RF Distance	0.4 cM	1.6 cM	1.1 cM	0.3 cM	2.3 cM

Table Appendix 4B: **:** Marker distances according to recombination frequencies (RF) of F_3 mapping population for barley 7H chromosome following recombinant screening

6.5. Appendix 5: Observing phenotypic resistant/genetically susceptible individuals in disease tests

Sample ID	**VIS**	**M15**	**M21**	**Progeny**
2.16	1	v	v	Fully susceptible
2.36	1	v	v	Fully susceptible
2.56	0	v	v	Fully susceptible

Appendix 5: Segregation of genetically susceptible individuals

6.6. Appendix 6 B: Heterozygous families used for Recombinant screening

Sample	M2	M6	F_2 VIS
2.27	h	h	5
2.58	h	h	21
2.60	h	h	8
2.21	v	h	24
2.18	h	h	15
2.14	h	h	18
2.54	h	h	24
2.43	h	h	0
2.3	h	h	0

Appendix 6A: 1H heterozygous individuals selfed to receive segregating families

Sample	M15	M21	F_2 VIS
2.40	h	h	1
2.11	h	h	0
2.1	h	h	2
2.5	h	h	0
2.15	h	h	0
2.19	h	h	1
2.4	h	h	0
2.5	h	h	0
2.31	h	h	1
2.19	h	h	1
2.42	s	h	0

Appendix 6B: 7H heterozygous individuals selfed to receive segregating families

6.7. Appendix 7: Identified *Rpgaq1* (segregating) Recombinants following screening

	Marker	M2	M4	J1	J2	J3	J4	J5	M6
	SNP ID	548	177	869	483	637	264	42	643
	Sample								
1	2.14.14	s	h	h	h	h	h	h	h
2	2.14.10	s	h	h	h	h	h	h	h
3	2.27.35	s	s	h	h	h	h	h	h
4	2.60.22	s	s	h	h	h	h	h	h
5	2.54.48	s	s	h	h	h	h	h	h
6	2.3.12	s	s	h	h	h	h	h	h
7	2.27.8	s	s	s	h	h	h	h	h
8	2.18.2	s	s	s	h	h	h	h	h
9	2.54.59	s	s	s	h	h	h	h	h
10	2.14.24	s	s	s	s	h	h	h	h
11	2.54.38	s	s	s	s	s	s	h	h
12	2.58.1	s	s	s	s	s	s	h	h
13	2.43.34	s	s	s	s	s	s	s	h
14	2.58.21	s	s	s	s	s	s	s	h
15	2.60.24	h	s	s	s	s	s	s	s
16	2.27.52	h	s	s	s	s	s	s	s
17	2.60.68	h	h	s	s	s	s	s	s
18	2.18.19	h	h	s	s	s	s	s	s
19	2.43.35	h	h	s	s	s	s	s	s
20	2.43.51	h	h	s	s	s	s	s	s
21	2.3.38	h	h	unknown	s	s	s	s	s
22	2.14.16	h	h	h	h	h	s	s	s

Figure Appendix 7: *Rpgaq1* segregating sh recombinants identified following screening progeny of heterozygous parents for *Rpgaq1* on barley 1H chromosome. Phenotype S=susceptible and R=resistant (not reliable because only pustules were observed). Framed samples were used for seedling test (3.5)

6.8. Appendix 8: Identified *Rpgaq1* (genetically resistant) Recombinants following screening

	Marker	M2	M4	J1	J2	J3	J4	J5	M6
	SNP ID	548	177	869	483	637	264	42	643
	Sample								
1	2.43.59	v	h	h	h	h	h	h	h
2	2.3.21	v	h	h	h	h	h	h	h
3	2.27.9	v	v	h	h	h	h	h	h
4	2.27.59	v	v	h	h	h	h	h	h
5	2.60.1	v	v	h	h	h	h	h	h
6	2.60.13	v	v	h	h	h	h	h	h
7	2.14.64	v	v	h	h	h	h	h	h
8	2.14.73	v	v	h	h	h	h	h	h
9	2.43.47	v	v	h	h	h	h	h	h
10	2.43.6	v	v	h	h	h	h	h	h
11	2.60.36	v	v	v	h	h	h	h	h
12	2.60.59	v	v	v	h	h	h	h	h
13	2.27.37	v	v	v	h	h	h	h	h
14	2.18.9	h	v	v	v	v*	v	v	v
15	2.18.29	h	v	v	v	v	v	v	v
16	2.54.36	h	v	v	v	v	v	v	v
17	2.43.73	h	v	v	v	v	v	v	v
18	2.14.63	h	h	v	v	v	v	v	v
19	2.54.55	h	h	v	v	v	v	v	v
20	2.3.62	h	h	v	v	v	v	v	v
21	2.43.61	h	h	v	v	v	v	v	v
22	2.27.4	h	h	v	v*	v	v	v	v
23	2.58.2	h	h	h	v	v	v	v	v
24	2.60.47	h	h	h	v	v	v	v	v
25	2.58.51	h	h	h	v	v	v	v	v

Figure Appendix 8: *Rpgaq1* resistant recombinants identified following screening progeny of heterozygous parents for barley 1H chromosome.

6.9. Appendix 9: Identified *Rpgaq5* (segregating) Recombinants following screening

Marker	M15	M17	J6	J7	J8	M21
SNP ID	28	095	557	631	396	615
Sample						
2.5.42	h	h	h	h	h	v
2.4.48	h	h	h	v	v	v
2.11.33	h	h	h	v	v	v
2.40.1	h	h	v	v	v	v
2.11.7	h	h	v	v	v	v
2.19.2	h	h	v	v	v	v
2.5.27	h	h	v	v	v	v
2.4.70	v	v	v	v	v	h
2.4.71	v	v	v	v	v	h
2.19.13	v	v	v	v	v	h
2.4.43	v	v	v	v	v	h
2.31.16	v	v	v	v	v	h
2.40.30	v	v	v	v	h	h
2.11.11	v	v	v	h	h	h
2.11.2	v	v	h	h	h	h
2.31.27	v	v	h	h	h	h

Figure Appendix 9: segregating vh recombinants identified following screening progeny of heterozygous parents for *Rpgaq5* on barley 7H chromosome. Phenotype S=susceptible and R=resistant (not reliable because only pustules were observed). Framed samples were used for seedling test (3.5)

6.10. Appendix 10: Identified *Rpgaq1* (genetically resistant) Recombinants following screening

Marker	M15	M17	J6	J7	J8	M21
SNP ID	28	095	557	631	396	615
2.19.30	s	s	h	h	h	h
2.4.47	s	s	h	h	h	h
2.1.15	s	s	h	h	h	h
2.40.5	s	s	h	h	h	h
2.19.12	s	s	s	h	h	h
2.15.43	s	s	s	unkown	h	h
2.19.6	s	s	s	s	h	h
2.19.11	s	s	s	s	s	h
2.15.25	s	s	s	s	s	h
2.5.6	s	s	s	s	s	h
2.19.34	s	s	s	s	s	h
2.5.33	h	h	s	s	s	s
2.40.7	h	h	h	s	s	s
2.15.22	h	h	h	s	s	s
2.40.23	h	h	h	s	s	s
2.4.56	h	h	h	s	s	s
2.5.37	h	h	h	unknown	s	s
2.1.9	h	h	h	h	h	s

Figure Appendix 10: resistant recombinants identified following screening progeny of heterozygous parents for barley 7H chromosome.

6.11. Appendix 11: 1st Seedling Test for *Rpgaq1* (1H)

A

Sample	Pustules	M2	M6	M7
2.38.1	0	h	h	v
2.38.2	1	h	h	v
2.38.4	0	h	h	v
2.38.9	1	h	h	v
2.38.10	0	h	h	v
2.38.12	0	h	h	v
2.38.13	0	h	h	v
2.38.14	0	h	unknown	v
2.38.21	1	h	h	v
2.38.23	0	h	h	v
2.38.24	0	h	h	v
2.38.25	0	h	h	v
2.38.26	0	h	h	v
2.38.27	0	h	h	v
2.38.5	25	s	s	v
2.38.6	3	s	s	v
2.38.7	17	s	s	v
2.38.16	6	s	s	v
2.38.20	29	s	s	v
2.38.22	0	s	s	v
2.38.28	1	s	unknown	v
2.38.29	6	s	s	v
2.38.30	3	s	s	v
2.38.15	1	unknown	unknown	v
2.38.3	0	v	v	v
2.38.8	0	v	v	v
2.38.11	0	v	v	v
2.38.17	0	v	v	v
2.38.18	0	v	v	v
2.38.19	0	v	v	v

C

Sample	Pustules	M1	M2	M6
2.49.1	1	v	h	h
2.49.2	0	v	h	h
2.49.4	2	v	h	h
2.49.5	1	v	h	h
2.49.7	4	v	h	h
2.49.8	2	v	h	h
2.49.9	2	v	h	h
2.49.10	1	v	h	h
2.49.11	4	v	h	h
2.49.14	1	v	h	h
2.49.17	0	v	h	h
2.49.18	1	v	h	h
2.49.19	4	v	h	h
2.49.20	3	v	h	h
2.49.21	1	v	h	unknown
2.49.25	4	v	h	unknown
2.49.27	2	v	h	h
2.49.28	2	v	h	h
2.49.29	0	v	h	h
2.49.3	10	v	s	s
2.49.6	24	v	s	s
2.49.15	6	v	s	s
2.49.16	11	v	s	s
2.49.22	7	v	s	unknown
2.49.26	18	v	s	s
2.49.13	0	v	v	v
2.49.23	0	v	v	v
2.49.24	0	v	v	v
2.49.12	0	v	v	v
2.49.30	0	v	v	v

B

VV compared to SS		
	Variable 1	*Variable 2*
Mittelwert	10,07	0,17
Varianz	114,10	0,03
Beobachtu	9,00	6,00
Hypothetis	0,00	
Freiheitsgr	8,00	
t-Statistik	2,78	
P(T<=t) ein	0,01	
Kritischer t	1,86	
P(T<=t) zw	0,02	
Kritischer t	2,31	
Expected		7,25
		14,5
		7,25
Ovserved		9
		14
		6
CHI SQ		0,720661

D

VV compared to SS		
	Variable 1	*Variable 2*
Mittelwert	0,000	12,556
Varianz	0,000	48,119
Beobachtu	5,000	6,000
Hypothetis	0,000	
Freiheitsgr	5,000	
t-Statistik	-4,434	
P(T<=t) ein	0,003	
Kritischer t	2,015	
P(T<=t) zw	0,007	
Kritischer t	2,571	
Expected		7,5
		15
		7,5
Ovserved		6
		19
		5
CHI SQ		0,332871

Appendix 11: A 2.38 progeny mapping *Rpgaq1* grouped according to M2 and M6 genotype segregation. **B**: Average VIS/cm leaf according to the genotype group and Chi –Square Test to detect segregation distortion. **C:** 2.49 progeny mapping *Rpgaq1* grouped according to M2 and M6 genotype segregation **D** Average VIS/cm leaf according to the genotype group and Chi –Square Test to detect segregation distortion.

6.12. Appendix 12: 1st Seedling Test for *Rpgaq5* (7H)

			SNP	
Sample		Pustules/ leaf	M15	M21
2-70 (7H)	11	0	s	s
	7	0	s	s
	19	0	s	s
	1	2	s	s
	4	0	h	s
	5	0	h	s
	6	1	h	s
	9	0	h	s
	10	0	h	s
	12	0	h	s
	14	0	h	s
	15	0	h	s
	17	0	h	s
	20	0	h	s
	21	0	h	s
	22	0	h	s
	23	0	h	s
	2	2	v	s
	3	0	v	s
	8	13	v	s
	13	4	v	s
	16	7	v	s
	18	1	v	s
	24	3	v	s

VV compared to SS		
	Variable 1	*Variable 2*
Mittelwert	0,416667	4,333333
Varianz	0,694444	19,18519
Beobachtu	4	7
Hypothetis	0	
Freiheitsgra	7	
t-Statistik	-2,29428	
P(T<=t) ein	0,027728	
Kritischer t	1,894579	
P(T<=t) zwe	0,055457	
Kritischer t	2,364624	
	Expected	6
		12
		6
	Ovserved	4
		13
		7
	CHI SQ	0,632337

Appendix 12: A 2.70 progeny mapping *Rpgaq5* grouped according to M15 genotype segregation. **B**: Average VIS/cm leaf according to the genotype group and Chi –Square Test to detect segregation distortion.

6.13. Appendix 13 Identified homozygous recombinants following genotyping of seedling test

6.13.1. Appendix 13 A: *Rpgaq1* homozygous recombinants

		M2	M4	J2	J5	M6	
		548	177	483	42	643	Phenotype
	Sample		55.6	66.5	67.1	70.8	
1	2.61.26	v	v	Unknown	Unknown	s	0 pustules
	2.12.10	s	Unknown	Unknown	Unknown	v	0 pustules
	2.27.8.13	s	s	v	v	v	0.00 VIS/cm leaf
	2.27.8.14	s	s	v	v	v	0.00 VIS/cm leaf
2	2.27.8.17	s	s	v	v	v	0.00 VIS/cm leaf
	2.58.32.2	s	s	s	s	v	2.36 VIS/cm leaf
	2.58.32.3	s	s	s	s	v	1.38 VIS/cm leaf
	2.58.32.9	s	s	s	s	v	0.47 VIS/cm leaf
	2.60.68.2	v	v	v	v	s	0.00 VIS/cm leaf
	2.60.68.8	v	v	v	v	s	1.09 VIS/cm leaf
	2.60.68.16	v	v	v	v	s	0.17 VIS/cm leaf
3	2.54.48.2	s	s	v	v	v	0 VIS/cm^2
	2.54.59.1	s	s	v	v	v	0 VIS/cm^2
	2.14.10.1	s	v	v	v	v	0 VIS/cm^2
	2.14.16.10	v	v	v	s	s	0 VIS/cm^2

Table Appendix 13A: identified barely 1H homozygous recombinants following seedling test using *Puccinia graminis* f. sp. avenae isolate Evertsholm. 1: first seedling test, 2: second seedling test, 3: third seedling test.

6.13.2. Appendix 13 B: *Rpgaq5* homozygous recombinants

		M15	J6	M21	
		028	557	615	VIS
	Sample	6.0	7.5	10.6	(per cm leaf)
1	2.12.15	s	Unknown	v	3 Pustules
	2.53.6	s	Unknown	V	0 Pustules
	2.69.27	v	Unknown	s	0 Pustules
	2.70.8	v	Unknown	s	13 Pustules
2	2.40.1.1	s	v	v	0.1 VIS/cm leaf
	2.40.1.3	s	v	v	0.0 VIS/cm leaf
	2.40.1.4	s	v	v	0.0 VIS/cm leaf
	2.11.7.4	s	v	v	0.2 VIS/cm leaf
	2.11.7.12	s	v	v	0.0 VIS/cm leaf
	2.11.11.2	v	v	s	0.5 VIS/cm leaf
	2.11.11.9	v	v	s	0.0 VIS/cm leaf
	2.11.11.18	v	v	s	0.2 VIS/cm leaf
	2.11.2.4	v	Unknown	s	0.2 VIS/cm leaf
	2.11.2.6	v	Unknown	s	0.2 VIS/cm leaf
	2.11.2.14	v	Unknown	s	0.1 VIS/cm leaf

Table Appendix 13B : identified barely 7H false homozygous recombinants following seedling test using *Puccinia graminis* f. sp. avenae isolate Evertsholm. 1: first seedling test, 2: second seedling test, 3: third seedling test.

6.14. Appendix 14: selected recombinants for 2nd seedling test to fine map *Rpgaq1*

	M2	M4	J1	J2	M6
Sample	548	177	869	483	643
2.27.35	s	s	h	h	h
2.27.8	s	s	s	h	h
2.58.32	s	s	s	s	h
2.60.68	h	h	s	s	s

Appendix 20: Collection of recombinants with unique recombinations in target region. Selected from 1H recombinant pool Appendix 7. Families were subjected to disease test with *Pga* Evertsholm to elucidate *Rpgaq1* based on family segregation.

6.15. Appendix 15: 2nd seedling test to fine map *Rpgaq1*

A

			VIS	
Sample	M4	J2	per leaf	(per cm leaf)
2.27.35.1	s	h	1	0,09
2.27.35.2	s	h	0	0,00
2.27.35.3	s	h	2	0,12
2.27.35.4	s	h	0	0,00
2.27.35.5	s	h	0	0,00
2.27.35.7	s	h	1	0,07
2.27.35.8	s	h	0	0,00
2.27.35.10	s	h	0	0,00
2.27.35.12	s	h	0	0,00
2.27.35.14	s	h	0	0,00
2.27.35.16	s	h	0	0,00
2.27.35.17	s	h	0	0,00
2.27.35.19	s	h	0	0,00
2.27.35.20	s	h	1	0,06
2.27.35.21	s	h	0	0,00
2.27.35.22	s	h	2	0,15
2.27.35.23	s	h	0	0,00
2.27.35.6	s	s	0	0,00
2.27.35.11	s	s	1	0,08
2.27.35.9	s	v	0	0,00
2.27.35.13	s	v	0	0,00
2.27.35.15	s	v	0	0,00
2.27.35.18	s	v	0	0,00
2.27.35.24	s	v	0	0,00

B

Average VIS/cm leaf	
v	0,000
h	0,029
s	0,033
CHI SQ Test	
ex	
vv	6
hh	12
ss	6
ob	
vv	5
hh	17
ss	2
Chi	0,086

Appendix 15A: A 2.27.35 progeny mapping *Rpgaq1* grouped according to J2 genotype segregation. **B**: Average VIS/cm leaf according to the genotype group and Chi –Square Test to detect segregation distortion.

			VIS	
Sample	M4	M6	per leaf	(per cm leaf)
2.58.32.1	s	s	33	2,64
2.58.32.2	s	s	26	2,36
2.58.32.3	s	s	11	1,38
2.58.32.4	s	s	18	1,50
2.58.32.5	s	s	25	2,27
2.58.32.6	s	s	16	1,33
2.58.32.7	s	s	33	2,75
2.58.32.8	s	s	11	1,22
2.58.32.9	s	s	7	0,47
2.58.32.10	s	s	9	0,67
2.58.32.11	s	s	6	0,80
2.58.32.12	s	s	9	0,64
2.58.32.13	s	s	18	3,00
2.58.32.14	s	s	17	1,48
2.58.32.15	s	s	4	0,47
2.58.32.16	s	s	5	0,59
2.58.32.17	s	s	8	0,80
2.58.32.18	s	s	11	1,00
2.58.32.19	s	s	10	0,80
2.58.32.20	s	s	19	1,46
2.58.32.21	s	s	20	1,82
2.58.32.22	s	s	6	0,60
2.58.32.23	s	s	13	1,30
2.58.32.24	s	s	10	1,00

Appendix 15B: 2.58.32 progeny and VIS per leaf and cm leaf

A

			VIS	
Sample	M4	J2	per leaf	per cm lea
2.27.8.1	s	h	0	0,00
2.27.8.2	s	h	0	0,00
2.27.8.3	s	h	2	0,16
2.27.8.5	s	h	1	0,08
2.27.8.7	s	h	2	0,12
2.27.8.9	s	h	1	0,06
2.27.8.11	s	h	14	0,97
2.27.8.15	s	h	0	0,00
2.27.8.16	s	h	0	0,00
2.27.8.18	s	h	2	0,14
2.27.8.4	s	s	0	0,00
2.27.8.6	s	s	0	0,00
2.27.8.8	s	s	1	0,08
2.27.8.10	s	s	3	0,20
2.27.8.12	s	s	1	0,08
2.27.8.13	s	v	0	0,00
2.27.8.14	s	v	0	0,00
2.27.8.17	s	v	0	0,00

B

Average VIS/cm leaf	
v	0,12
h	0,13
s	0,06
CHI SQ Test	
ex	
vv	4,50
hh	9,00
ss	4,50
ob	
vv	3,00
hh	10,00
ss	5,00
Chi	0,72

Appendix 15C: A 2.27.8 progeny mapping *Rpgaq1* grouped according to J2 genotype segregation. **B**: Average VIS/cm leaf according to the genotype group and Chi –Square Test to detect segregation distortion.

A

Sample	M4	J2	VIS per leaf	VIS (per cm leaf)
2.60.68.1	h	s	2	0,20
2.60.68.4	h	s	17	1,21
2.60.68.5	h	s	0	0,00
2.60.68.9	h	s	1	0,07
2.60.68.11	h	s	1	0,08
2.60.68.14	h	s	13	1,24
2.60.68.15	h	s	5	0,45
2.60.68.17	h	s	4	0,36
2.60.68.6	s	s	0	0,00
2.60.68.10	s	s	1	0,07
2.60.68.12	s	s	2	0,14
2.60.68.13	s	s	2	0,18
2.60.68.2	v	s	0	0,00
2.60.68.3	v	s	0	0,00
2.60.68.7	v	s	6	0,43
2.60.68.8	v	s	12	1,09
2.60.68.16	v	s	2	0,17
2.60.68.18	v	s	1	0,08

B

Average VIS/cm leaf	
v	0,53
h	0,14
s	0,37
CHI SQ Test	
ex	
vv	4,5
hh	9
ss	4,5
ob	
vv	6
hh	8
ss	4

Appendix 15D: A 2.60.68 progeny mapping *Rpgaq1* grouped according to M4 genotype segregation. **B**: Average VIS/cm leaf according to the genotype group and Chi –Square Test to detect segregation distortion.

6.16. Appendix 16: selected recombinants for 2nd seedling test to fine map *Rpgaq5*

	M15	M17	J6	J7	J8	M21
Sample	028	095	557	631	396	615
2.40.1	h	h	h	v	v	v
2.11.7	h	h	v	v	v	v
2.11.11	v	v	v	h	h	h
2.11.2	v	v	h	h	h	h

Appendix 16: Collection of recombinants with unique recombinations in target region. Families were subjected to disease test with *Pga* Evertsholm to elucidate *Rpgaq5* based on family segregation.

6.17. Appendix 17: 2nd seedling test to fine map *Rpgaq5*

A

			VIS	
Sample	M17	J6	per leaf	per cm lea
2.11.7.2	h	v	0	0,0
2.11.7.3	h	v	1	0,1
2.11.7.5	h	v	0	0,0
2.11.7.6	h	v	0	0,0
2.11.7.10	h	v	0	0,0
2.11.7.11	h	v	0	0,0
2.11.7.13	h	v	0	0,0
2.11.7.14	h	v	0	0,0
2.11.7.4	s	v	3	0,2
2.11.7.9	s	v	0	0,0
2.11.7.12	s	v	0	0,0
2.11.7.7	v	v	1	0,1
2.11.7.8	v	v	1	0,1
2.11.7.1	v	v	4	0,3

B

Average VIS/cm leaf	
v	0,15
h	0,01
s	0,07
CHI SQ Test	
ex	
vv	3,5
hh	7
ss	3,5
ob	
vv	3
hh	8
ss	3
Chi	0,87

Appendix 17A: A 2.11.7 progeny mapping *Rpgaq5* grouped according to M17genotype segregation. **B**: Average VIS/cm leaf according to the genotype group and Chi –Square Test to detect segregation distortion.

A

Sample	M17	J8	VIS per leaf	VIS (per cm leaf)
2.11.11.1	v	h	5	0,455
2.11.11.5	v	h	1	0,125
2.11.11.6	v	h	2	0,200
2.11.11.7	v	h	2	0,200
2.11.11.8	v	h	4	0,381
2.11.11.10	v	h	1	0,087
2.11.11.11	v	h	4	0,471
2.11.11.12	v	h	3	0,375
2.11.11.14	v	h	0	0,000
2.11.11.15	v	h	3	0,353
2.11.11.17	v	h	9	0,067
2.11.11.2	v	s	6	0,500
2.11.11.9	v	s	0	0,000
2.11.11.18	v	s	2	0,200
2.11.11.4	v	unknown	2	0,182
2.11.11.3	v	v	3	0,400
2.11.11.13	v	v	3	0,273
2.11.11.16	v	v	1	0,087
2.11.11.19	v	v	5	0,455

B

Average VIS/cm leaf	
v	0,304
h	0,247
s	0,233
CHI SQ Test	
ex	
vv	4,75
hh	9,5
ss	4,75
ob	
vv	4
hh	11
ss	3
Chi	0,61

Appendix 17B: A 2.11.11 progeny mapping *Rpgaq5* grouped according to M17genotype segregation. **B**: Average VIS/cm leaf according to the genotype group and Chi –Square Test to detect segregation distortion.

A

Sample	M17	J6	VIS per leaf	VIS (per cm leaf)
2.11.2.10	v	h	*	*
2.11.2.18	v	h	12	0,96
2.11.2.3	v	h	1	0,10
2.11.2.5	v	h	2	0,17
2.11.2.8	v	h	2	0,12
2.11.2.11	v	h	3	0,22
2.11.2.12	v	h	1	0,09
2.11.2.13	v	h	0	0,00
2.11.2.16	v	h	2	0,14
2.11.2.19	v	h	3	0,27
2.11.2.20	v	h	1	0,09
2.11.2.23	v	h	0	0,00
2.11.2.24	v	h	0	0,00
2.11.2.4	v	s	3	0,20
2.11.2.6	v	s	2	0,17
2.11.2.14	v	s	1	0,09
2.11.2.2	v	v	8	0,84
2.11.2.7	v	v	5	0,45
2.11.2.9	v	v	3	0,25
2.11.2.15	v	v	2	0,17
2.11.2.17	v	v	1	0,07
2.11.2.21	v	v	4	0,28
2.11.2.22	v	v	3	0,25
2.11.2.1	v	v	8	0,50

B

Average VIS/cm leaf	
v	0,35
h	0,18
s	0,15
CHI SQ Test	
ex	
vv	6
hh	12
ss	6
ob	
vv	8
hh	13
ss	3
Chi	0,32

Appendix 17C: A 2.11.2 progeny mapping *Rpgaq5* grouped according to M17genotype segregation. **B**: Average VIS/cm leaf according to the genotype group and Chi –Square Test to detect segregation distortion.

6.18. Appendix 18: 3rd seedling test to fine map *Rpgaq1*

A

Sample	VIS/cm2	548 (M2)	643 (M6)
2.14.10.1	0,00	s	v
2.14.10.4	0,37	s	v
2.14.10.10	0,00	s	v
2.14.10.11	0,00	s	v
2.14.10.12	0,00	s	v
2.14.10.14	0,00	s	v
2.14.10.15	0,00	s	v
2.14.10.16	0,12	s	v
2.14.10.25	0,00	s	v
2.14.10.27	0,00	s	v
2.14.10.28	0,00	s	v
2.14.10.34	0,00	s	v
2.14.10.2	0,20	s	h
2.14.10.3	0,28	s	h
2.14.10.6	0,00	s	h
2.14.10.7	0,00	s	h
2.14.10.8	0,00	s	h
2.14.10.9	0,00	s	h
2.14.10.17	1,14	s	h
2.14.10.18	0,00	s	h
2.14.10.19	0,00	s	h
2.14.10.20	0,24	s	h
2.14.10.21	0,14	s	h
2.14.10.23	0,11	s	h
2.14.10.24	0,14	s	h
2.14.10.29	0,00	s	h
2.14.10.32	0,20	s	h
2.14.10.33	0,00	s	h
2.14.10.35	0,00	s	h
2.14.10.36	0,00	s	h
2.14.10.5	8,52	s	s
2.14.10.13	5,58	s	s
2.14.10.22	3,19	s	s
2.14.10.26	4,72	s	s
2.14.10.30	5,65	s	s
2.14.10.31	5,34	s	s
152.1	5,56		
152.2	4,47		
152.3	5,63		
152.4	4,92		
152.5	7,86		
110.1	0,00		
110.2	0,00		
110.3	0,00		
110.4	0,00		
110.5	0,00		

B

	Avg. VIS	Avg VIS/cm	Avg VIS/cm2
RIL 110	0	0,00	0
vv	0,33	0,03	0,04
hh	0,89	0,22	0,14
ss	38,83	1,36	5,50
RIL 152	27,2	3,22	5,69
Expected	vv	9	
	hh	18	
	ss	9	
Observed	v	12	
	h	18	
	s	6	
CHI		0,37	

C

SS compared to RIL 152

Zweistichproben t-Test unter der Annahme

	Variable 1	*Variable 2*
Mittelwert	5,501	5,688
Varianz	3,022	1,697
Beobachtunge	6,000	5,000
Hypothetische	0,000	
Freiheitsgrade	9,000	
t-Statistik	-0,203	
P(T<=t) einsei	0,422	
Kritischer t-W	1,833	
P(T<=t) zweise	0,843	
Kritischer t-W	2,262	

VV compared to SS

Zweistichproben t-Test unter der Annahme

	Variable 1	*Variable 2*
Mittelwert	0,041	5,501
Varianz	0,012	3,022
Beobachtunge	12,000	6,000
Hypothetische	0,000	
Freiheitsgrade	5,000	
t-Statistik	-7,687	
P(T<=t) einsei	0,000	
Kritischer t-W	2,015	
P(T<=t) zweise	0,001	
Kritischer t-W	2,571	

HH compared to VV

Zweistichproben t-Test unter der Annahme

	Variable 1	*Variable 2*
Mittelwert	0,041	0,136
Varianz	0,012	0,072
Beobachtunge	12,000	18,000
Hypothetische	0,000	
Freiheitsgrade	24,000	
t-Statistik	-1,343	
P(T<=t) einsei	0,096	
Kritischer t-W	1,711	
P(T<=t) zweise	0,192	
Kritischer t-W	2,064	

Appendix 18: A 2.14.10 progeny mapping *Rpgaq1* grouped according to M6 genotype segregation. VIS/cm^2 is color shaded green= low infection and red=high infection. **B**: Average VIS/cm^2 according to the genotype group and Chi –Square Test to detect segregation distortion. **C:** Student's T Test to compare means between Susceptible SS and positive control RIL 152, test VV compared to SS, and test HH compared to VV. ($p<0.05$)

6.19. Appendix 19

A

Sample	VIS/cm^2	548 (M2)	643 (M6)
2.54.48.1	0,00	s	v
2.54.48.2	0,00	s	v
2.54.48.6	0,00	s	v
2.54.48.17	0,00	s	v
2.54.48.22	0,00	s	v
2.54.48.24	0,13	s	v
2.54.48.29	0,12	s	v
2.54.48.30	0,00	s	v
2.54.48.32	0,00	s	v
2.54.48.36	0,10	s	v
2.54.48.3	0,19	s	h
2.54.48.4	0,28	s	h
2.54.48.7	0,00	s	h
2.54.48.8	0,00	s	h
2.54.48.9	0,48	s	h
2.54.48.10	0,00	s	h
2.54.48.11	0,00	s	h
2.54.48.12	0,58	s	h
2.54.48.13	0,52	s	h
2.54.48.14	0,00	s	h
2.54.48.15	0,12	s	h
2.54.48.16	0,48	s	h
2.54.48.18	0,00	s	h
2.54.48.19	0,00	s	h
2.54.48.21	0,24	s	h
2.54.48.23	0,20	s	h
2.54.48.25	0,00	s	h
2.54.48.26	0,23	s	h
2.54.48.27	0,23	s	h
2.54.48.31	0,00	s	h
2.54.48.33	0,20	s	h
2.54.48.34	0,00	s	h
2.54.48.35	0,29	s	h
2.54.48.5	12,69	s	s
2.54.48.20	11,10	s	s
2.54.48.28	12,33	s	s
152.1	6,12		
152.2	5,18		
152.3	6,12		
152.4	3,90		
152.5	9,00		
110.1	0,00		
110.2	0,00		
110.3	0,00		
110.4	0,00		
110.5	0,00		

B

	Avg VIS	Avg VIS / cm	VIS/cm2
RIL110	0,00	0,00	0,00
vv	1,20	0,10	0,10
hh	3,00	0,25	0,17
ss	82,67	7,00	12,04
RIL 152	30,00	3,25	6,06
Expected		v	9
		h	18
		s	9
Observed		v	10
		h	23
		s	3
CHI			0,06

C

SS compared to RIL 152

Zweistichproben t-Test unter der Annahme

	Variable 1	*Variable 2*
Mittelwert	12,039	6,0625341
Varianz	0,694	3,52846538
Beobachtunge	3,000	5
Hypothetische	0,000	
Freiheitsgrade	6,000	
t-Statistik	6,174	
P(T<=t) einsei	0,000	
Kritischer t-W	1,943	
P(T<=t) zweise	0,001	
Kritischer t-W	2,447	

VV compared to SS

Zweistichproben t-Test unter der Annahme u

	Variable 1	*Variable 2*
Mittelwert	0,035	12,039
Varianz	0,003	0,694
Beobachtunge	10,000	3,000
Hypothetische	0,000	
Freiheitsgrade	2,000	
t-Statistik	-24,935	
P(T<=t) einsei	0,001	
Kritischer t-W	2,920	
P(T<=t) zweise	0,002	
Kritischer t-W	4,303	

HH compared to VV

Zweistichproben t-Test unter der Annahme u

	Variable 1	*Variable 2*
Mittelwert	0,035	0,174
Varianz	0,003	0,037
Beobachtunge	10,000	23,000
Hypothetische	0,000	
Freiheitsgrade	29,000	
t-Statistik	-3,184	
P(T<=t) einsei	0,002	
Kritischer t-W	1,699	
P(T<=t) zweise	0,003	
Kritischer t-W	2,045	

Appendix 19: A 2.54.48 progeny mapping *Rpgaq1* grouped according to M6 genotype segregation. VIS/cm^2 is color shaded green= low infection and red=high infection. **B**: Average VIS/cm^2 according to the genotype group and Chi –Square Test to detect segregation distortion. **C:** Student's T Test to compare means between Susceptible SS and positive control RIL 152, test VV compared to SS, and test HH compared to VV. ($p<0.05$)

6.20. Appendix 20

A

Sample	VIS/cm²	548 (M2)	643 (M6)
2.54.59.1	0,00	s	v
2.54.59.13	0,09	s	v
2.54.59.15	0,11	s	v
2.54.59.5	0,10	s	v
2.54.59.11	0,12	s	v
2.54.59.27	0,09	s	v
2.54.59.7	0,31	s	v
2.54.59.10	0,31	s	v
2.54.59.4	0,34	s	v
2.54.59.29	0,25	s	v
2.54.59.2	0,33	s	v
2.54.59.8	0,45	s	v
2.54.59.22	0,00	s	h
2.54.59.32	0,00	s	h
2.54.59.41	0,19	s	h
2.54.59.39	0,13	s	h
2.54.59.14	0,21	s	h
2.54.59.31	0,30	s	h
2.54.59.12	0,30	s	h
2.54.59.40	0,31	s	h
2.54.59.36	0,34	s	h
2.54.59.37	0,34	s	h
2.54.59.25	0,36	s	h
2.54.59.20	0,42	s	h
2.54.59.16	0,43	s	h
2.54.59.24	0,43	s	h
2.54.59.33	0,50	s	h
2.54.59.9	0,52	s	h
2.54.59.18	0,52	s	h
2.54.59.17	0,63	s	h
2.54.59.30	0,75	s	h
2.54.59.28	0,68	s	h
2.54.59.23	1,14	s	h
2.54.59.6	1,04	s	h
2.54.59.3	16,88	s	s
2.54.59.19	18,27	s	s
2.54.59.21	17,31	s	s
2.54.59.26	17,19	s	s
2.54.59.34	13,44	s	s
2.54.59.35	20,12	s	s
2.54.59.38	14,22	s	s
110.1	0,00		
110.2	0,12		
110.3	0,18		
110.4	0,00		
110.5	0,00		
152.1	5,00		
152.2	6,82		
152.3	8,06		
152.4	5,52		
152.5			

B

	VIS	VIS per cm	VIS/cm2
RIL 110	0,00	0,00	0,06
vv	2,42	0,20	0,21
hh	4,00	0,35	0,43
ss	145,29	11,50	16,77
RIL 152	29,00	4,14	6,35
	Expected	v	10,25
		h	21,5
		s	10,25
	Observed	v	12
		h	22
		s	7
	CHI		0,51

C

SS compared to RIL 152

Zweistichproben t-Test unter der Annahme

	Variable 1	*Variable 2*
Mittelwert	16,774	6,349
Varianz	5,254	1,878
Beobachtunge	7,000	4,000
Hypothetische	0,000	
Freiheitsgrade	9,000	
t-Statistik	9,438	
P(T<=t) einsei	0,000	
Kritischer t-W	1,833	
P(T<=t) zweise	0,000	
Kritischer t-W	2,262	

VV compared to SS

Zweistichproben t-Test unter der Annahme un

	Variable 1	*Variable 2*
Mittelwert	0,209	16,774
Varianz	0,020	5,254
Beobachtunge	12,000	7,000
Hypothetische	0,000	
Freiheitsgrade	6,000	
t-Statistik	-19,099	
P(T<=t) einsei	0,000	
Kritischer t-W	1,943	
P(T<=t) zweise	0,000	
Kritischer t-W	2,447	

HH compared to VV

Zweistichproben t-Test unter der Annahme un

	Variable 1	*Variable 2*
Mittelwert	0,209	0,433
Varianz	0,020	0,082
Beobachtunge	12,000	22,000
Hypothetische	0,000	
Freiheitsgrade	32,000	
t-Statistik	-3,054	
P(T<=t) einsei	0,002	
Kritischer t-W	1,694	
P(T<=t) zweise	0,005	
Kritischer t-W	2,037	

Appendix 20: A 2.54.59 progeny mapping *Rpgaq1* grouped according to M6 genotype segregation. VIS/cm² is color shaded green= low infection and red=high infection. **B**: Average VIS/cm² according to the genotype group and Chi –Square Test to detect segregation distortion. **C:** Student's T Test to compare means between Susceptible SS and positive control RIL 152, test VV compared to SS, and test HH compared to VV. ($p<0.05$)

6.21. Appendix 21

A

Sample	VIS/cm^2	548 (M2)	643(M6)
2.54.38.2	5,74	s	h
2.54.38.3	5,50	s	h
2.54.38.5	6,88	s	h
2.54.38.6	6,29	s	h
2.54.38.7	6,02	s	h
2.54.38.10	5,87	s	h
2.54.38.11	6,98	s	h
2.54.38.16	7,02	s	h
2.54.38.4	6,00	s	s
2.54.38.8	8,21	s	s
2.54.38.9	8,54	s	s
2.54.38.12	6,88	s	v
2.54.38.13	6,35	s	v
2.54.38.14	10,94	s	v
2.54.38.15	5,29	s	v
152.1	2,71		
152.2	3,33		
152.3	4,89		
152.4	4,66		
152.5	3,65		
110.1	0,00		
110.2	0,00		
110.3	0,12		
110.4	0,00		
110.4	0,20		

B

	VIS/cm2	
RIL 110	0,06	
vv	7,36	
hh	6,29	
ss	7,59	
RIL 152	3,85	
Expected	v	3,75
	h	7,5
	s	3,75
Observed	v	4
	h	8
	s	3
CHI		0,90

C

SS compared to RIL 152

Zweistichproben t-Test unter der Annahme

	Variable 1	*Variable 2*
Mittelwert	7,585	3,849
Varianz	1,912	0,837
Beobachtunge	3,000	5,000
Hypothetische	0,000	
Freiheitsgrade	3,000	
t-Statistik	4,166	
P(T<=t) einseit	0,013	
Kritischer t-W	2,353	
P(T<=t) zweise	0,025	
Kritischer t-W	3,182	

VV compared to SS

Zweistichproben t-Test unter der Annahme

	Variable 1	*Variable 2*
Mittelwert	7,362	7,585
Varianz	6,118	1,912
Beobachtunge	4,000	3,000
Hypothetische	0,000	
Freiheitsgrade	5,000	
t-Statistik	-0,152	
P(T<=t) einseit	0,443	
Kritischer t-W	2,015	
P(T<=t) zweise	0,885	
Kritischer t-W	2,571	

HH compared to VV

Zweistichproben t-Test unter der Annahme

	Variable 1	*Variable 2*
Mittelwert	6,287	7,585
Varianz	0,363	1,912
Beobachtunge	8,000	3,000
Hypothetische	0,000	
Freiheitsgrade	2,000	
t-Statistik	-1,571	
P(T<=t) einseit	0,128	
Kritischer t-W	2,920	
P(T<=t) zweise	0,257	
Kritischer t-W	4,303	

Appendix 21: A 2.54.38 progeny mapping *Rpgaq1* grouped according to M6 genotype segregation. VIS/cm^2 is color shaded green= low infection and red=high infection. **B**: Average VIS/cm^2 according to the genotype group and Chi –Square Test to detect segregation distortion. **C:** Student's T Test to compare means between Susceptible SS and positive control RIL 152, test VV compared to SS, and test HH compared to VV. ($p<0.05$)

6.22. Appendix 22

A

Sample	VIS/cm^2	548 (M2)	643 (M6)
2.43.34.1	3,33	s	s
2.43.34.2	0,76	s	s
2.43.34.3	2,46	s	s
2.43.34.4	0,30	s	s
2.43.34.5	0,60	s	s
2.43.34.6	3,33	s	s
2.43.34.7	0,56	s	s
2.43.34.8	1,27	s	s
2.43.34.9	1,30	s	s
2.43.34.10	1,82	s	s
2.43.34.11	0,36	s	s
2.43.34.12	0,61	s	s
2.43.34.13	1,19	s	s
2.43.34.14	0,73	s	s
2.43.34.15	2,18	s	s
2.43.34.16	1,09	s	s
2.43.34.17	1,98	s	s
2.43.34.18	6,94	s	s
2.43.34.19	0,55	s	s
2.43.34.20	1,39	s	s
2.43.34.21	2,59	s	s
2.43.34.22	0,55	s	s
2.43.34.23	1,67	s	s
2.43.34.24	2,40	s	s
2.43.34.25	1,21	s	s
2.43.34.26	0,61	s	s
2.43.34.27	2,00	s	s
2.43.34.28	0,99	s	s
2.43.34.29	0,00	s	s
2.43.34.30	0,18	s	s
2.43.34.31	1,41	s	s
2.43.34.32	2,48	s	s
2.43.34.33	1,10	s	s
2.43.34.34	0,52	s	s
2.43.34.35	4,10	s	s
2.43.34.36	2,67	s	s
2.43.34.37	0,95	s	s
2.43.34.38	1,03	s	s
2.43.34.39	0,38	s	s
2.43.34.40	0,71	s	s
2.43.34.41	0,64	s	s
2.43.34.42	2,74	s	s
2.43.34.43	0,55	s	s
2.43.34.44	1,80	s	s

1	152.1	1,71		
2	152.2	2,80		
3	152.3	0,89		
4	152.4	2,42		
1	110.1	0,00		
2	110.2	0,00		
3	110.3	0,00		
4	110.4	0,00		
5	110.5	0,00		

B

	avg VIS	avg VIS/cm	VIS/cm2
RIL 110	0,00	0	0
ss	9,48	0,82546073	1,50059146
RIL 152	10,75	1,03903319	1,95685426

IL 152 compared to SS grou	
Zweistichproben t-Test unte	
	Variable 1
Mittelwert	1,501
Varianz	1,627
Beobachtunge	44,000
Hypothetische	0,000
Freiheitsgrade	4,000
t-Statistik	-0,985
P(T<=t) einsei	0,190
Kritischer t-W	2,132
P(T<=t) zweise	0,380
Kritischer t-W	2,776

Appendix 22: A 2.43.34 progeny mapping *Rpgaq1* grouped according to M6 genotype segregation. VIS/cm^2 is color shaded green= low infection and red=high infection. **B**: Average VIS/cm^2 according to the genotype group and a Student' T Test comparing the means of RIL 152 and the ss genotype group.

6.23. Appendix 23

A

Sample	VIS/cm^2	548 (M2)	643 (M6)
2.14.16.10	0,12	s	v
2.14.16.11	0,00	s	v
2.14.16.16	0,00	s	v
2.14.16.23	0,12	s	v
2.14.16.26	0,00	s	v
2.14.16.34	0,00	s	v
2.14.16.35	0,22	s	v
2.14.16.37	0,00	s	v
2.14.16.38	0,00	s	v
2.14.16.40	0,00	s	v
2.14.16.14	0,00	s	h
2.14.16.22	0,00	s	h
2.14.16.27	0,00	s	h
2.14.16.29	0,00	s	h
2.14.16.30	0,00	s	h
2.14.16.36	0,00	s	h
2.14.16.31	0,10	s	h
2.14.16.25	0,11	s	h
2.14.16.12	0,14	s	h
2.14.16.13	0,14	s	h
2.14.16.15	0,14	s	h
2.14.16.19	0,47	s	h
2.14.16.39	0,00	s	h
2.14.16.3	0,00	s	h
2.14.16.5	0,00	s	h
2.14.16.2	0,00	s	h
2.14.16.18	1,43	s	h
2.14.16.17	1,82	s	h
2.14.16.1	4,44	s	s
2.14.16.4	4,63	s	s
2.14.16.6	5,11	s	s
2.14.16.7	3,00	s	s
2.14.16.8	6,29	s	s
2.14.16.9	2,73	s	s
2.14.16.20	1,47	s	s
2.14.16.21	0,60	s	s
2.14.16.28	1,14	s	s
2.14.16.32	2,17	s	s
2.14.16.33	2,32	s	s
2.14.16.41	1,07	s	s
2.14.16.42	1,46	s	s
2.14.16.43	1,76	s	s
2.14.16.24	4,80	s	s
152.1	6,60		
152.2	5,33		
152.3	5,19		
152.4	5,71		
152.5	6,67		
110.1	0,00		
110.2	0,00		
110.3	0,00		
110.4	0,00		
110.5	0,00		

B

Group	Avg VIS	Avg VIS / cm	VIS/cm2
RIL 110	0,00	0	0
vv	0,30	0,03	0,05
hh	2,33	0,21	0,24
ss	14,14	1,36	2,87
RIL 152	30,00	3,25	5,90
Expected	v	10,75	
	h	21,5	
	s	10,75	
Observed	v	10	
	h	18	
	s	14	
CHI		0,45	

C

SS compared to RIL 152

Zweistichproben t-Test unter der Annahme

	Variable 1	*Variable 2*
Mittelwert	2,865	5,902
Varianz	3,102	0,483
Beobachtunge	15,000	5,000
Hypothetische	0,000	
Freiheitsgrade	17,000	
t-Statistik	-5,513	
P(T<=t) einseit	0,000	
Kritischer t-W	1,740	
P(T<=t) zweise	0,000	
Kritischer t-W	2,110	

VV compared to SS

Zweistichproben t-Test unter der Annahme

	Variable 1	*Variable 2*
Mittelwert	0,047	2,865
Varianz	0,006	3,102
Beobachtunge	10,000	15,000
Hypothetische	0,000	
Freiheitsgrade	14,000	
t-Statistik	-6,188	
P(T<=t) einseit	0,000	
Kritischer t-W	1,761	
P(T<=t) zweise	0,000	
Kritischer t-W	2,145	

HH compared to VV

Zweistichproben t-Test unter der Annahme

	Variable 1	*Variable 2*
Mittelwert	0,047	0,242
Varianz	0,006	0,270
Beobachtunge	10,000	18,000
Hypothetische	0,000	
Freiheitsgrade	18,000	
t-Statistik	-1,556	
P(T<=t) einseit	0,069	
Kritischer t-W	1,734	
P(T<=t) zweise	0,137	
Kritischer t-W	2,101	

Appendix 23: A 2.14.16 progeny mapping *Rpgaq1* grouped according to M6 genotype segregation. VIS/cm^2 is color shaded green= low infection and red=high infection. **B**: Average VIS/cm^2 according to the genotype group and Chi –Square Test to detect segregation distortion. **C:** Student's T Test to compare means between Susceptible SS and positive control RIL 152, test VV compared to SS, and test HH compared to VV. ($p<0.05$)

6.24. Appendix 24

A

Sample	VIS/cm^2	548 (M2)	643 (M6)
2.14.24.3	0,97	s	v
2.14.24.4	1,00	s	v
2.14.24.5	1,00	s	v
2.14.24.6	1,06	s	v
2.14.24.7	1,25	s	v
2.14.24.8	1,28	s	v
2.14.24.11	1,29	s	v
2.14.24.13	1,32	s	v
2.14.24.16	1,87	s	v
2.14.24.21	2,08	s	v
2.14.24.22	2,24	s	v
2.14.24.23	2,36	s	v
2.14.24.35	3,17	s	v
2.14.24.41	4,00	s	v
2.14.24.1	0,14	s	h
2.14.24.2	0,14	s	h
2.14.24.15	0,19	s	h
2.14.24.17	0,23	s	h
2.14.24.18	1,11	s	h
2.14.24.20	1,33	s	h
2.14.24.24	0,00	s	h
2.14.24.26	0,36	s	h
2.14.24.27	0,16	s	h
2.14.24.29	0,32	s	h
2.14.24.30	1,22	s	h
2.14.24.31	1,26	s	h
2.14.24.32	1,53	s	h
2.14.24.34	1,88	s	h
2.14.24.36	1,90	s	h
2.14.24.38	1,91	s	h
2.14.24.39	2,32	s	h
2.14.24.25	2,96	s	h
2.14.24.28	2,96	s	h
2.14.24.33	3,06	s	h
2.14.24.9	2,56	s	s
2.14.24.10	3,00	s	s
2.14.24.12	3,27	s	s
2.14.24.14	0,46	s	s
2.14.24.19	0,70	s	s
2.14.24.37	0,77	s	s
2.14.24.40	0,92	s	s
41			
110.1	0,00		
100.2	0,00		
100.3	0,00		
100.4	0,00		
152.1	1,93		
152.2	2,28		
152.3	3,60		
152.4	5,15		
152.5	3,80		

B

	Avg VIS	Avg VIS / cm	VIS/cm^2
RIL 110	0	0	0
vv	3,83	0,30	1,78
hh	11,79	0,92	1,25
ss	12,52	1,00	1,67
RIL 152	19,00	1,84	3,35
Expected	v	10,25	
	h	20,5	
	s	10,25	
Observed	v	14	
	h	20	
	s	7	
CHI		0,30	

C

SS compared to RIL 152

Zweistichproben t-Test unter der Annahme unte

	Variable 1	*Variable 2*
Mittelwert	1,669	3,352
Varianz	1,487	1,668
Beobachtunge	7,000	5,000
Hypothetische	0,000	
Freiheitsgrade	8,000	
t-Statistik	-2,278	
P(T<=t) einsei	0,026	
Kritischer t-W	1,860	
P(T<=t) zweise	0,052	
Kritischer t-W	2,306	

VV compared to SS

Zweistichproben t-Test unter der Annahme

	Variable 1	*Variable 2*
Mittelwert	1,778	1,669
Varianz	0,836	1,487
Beobachtunge	14,000	7,000
Hypothetische	0,000	
Freiheitsgrade	10,000	
t-Statistik	0,209	
P(T<=t) einsei	0,419	
Kritischer t-W	1,812	
P(T<=t) zweise	0,839	
Kritischer t-W	2,228	

HH compared to VV

Zweistichproben t-Test unter der Annahme

	Variable 1	*Variable 2*
Mittelwert	1,778	1,248
Varianz	0,836	1,085
Beobachtunge	14,000	20,000
Hypothetische	0,000	
Freiheitsgrade	30,000	
t-Statistik	1,569	
P(T<=t) einsei	0,064	
Kritischer t-W	1,697	
P(T<=t) zweise	0,127	
Kritischer t-W	2,042	

Appendix 24: A 2.14.24 progeny mapping *Rpgaq1* grouped according to M6 genotype segregation. VIS/cm^2 is color shaded green= low infection and red=high infection. **B**: Average VIS/cm^2 according to the genotype group and Chi –Square Test to detect segregation distortion. **C:** Student's T Test to compare means between Susceptible SS and positive control RIL 152, test VV compared to SS, and test HH compared to VV. ($p<0.05$)

6.25. Appendix 25

A

Sample	VIS/cm^2	028 (M15)	557 (J6)
2.69.3	0,00	v	s
2.69.12	0,00	v	s
2.69.14	0,00	v	s
2.69.17	0,00	v	s
2.69.24	0,00	v	s
2.69.30	0,00	v	s
2.69.2	0,00	v	h
2.69.7	0,00	v	h
2.69.8	0,00	v	h
2.69.9	0,00	v	h
2.69.13	0,11	v	h
2.69.16	0,00	v	h
2.69.20	0,16	v	h
2.69.23	0,00	v	h
2.69.25	0,00	v	h
2.69.27	0,00	v	h
2.69.28	0,32	v	h
2.69.29	0,00	v	h
2.69.31	0,00	v	h
2.69.1	1,43	v	v
2.69.4	0,38	v	v
2.69.5	1,34	v	v
2.69.6	1,50	v	v
2.69.10	0,95	v	v
2.69.11	2,18	v	v
2.69.15	0,86	v	v
2.69.18	0,48	v	v
2.69.19	0,75	v	v
2.69.21	0,48	v	v
2.69.22	2,50	v	v
2.69.26	2,94	v	v
152.1	4,03		
152.2	8,57		
152.3	10,00		
152.4	9,14		
152.5	5,88		
143.1	0,10		
143.2	0,00		
143.3	0,00		
143.4	0,11		
143.5	0,12		
	VIS/cm2		
143	0,07		
ss	0,00		
hh	0,04		
vv	1,32		
152	7,52		
Expected	v	7,75	
	h	15,5	
	s	7,75	
Observed	v	12	
	h	13	
	s	6	
CHI		0,20918921	

B

C

VV compared to RIL 152		
Zweistichproben t-Test unter der Annahme		
	Variable 1	*Variable 2*
Mittelwert	1,316	7,525
Varianz	0,709	6,195
Beobachtunge	12,000	5,000
Hypothetische	0,000	
Freiheitsgrade	4,000	
t-Statistik	-5,450	
P(T<=t) einsei	0,003	
Kritischer t-W	2,132	
P(T<=t) zweise	0,006	
Kritischer t-W	2,776	
SS compared to VV		
Zweistichproben t-Test unter der Annahme		
	Variable 1	*Variable 2*
Mittelwert	0,000	1,316
Varianz	0,000	0,709
Beobachtunge	6,000	12,000
Hypothetische	0,000	
Freiheitsgrade	11,000	
t-Statistik	-5,412	
P(T<=t) einsei	0,000	
Kritischer t-W	1,796	
P(T<=t) zweise	0,000	
Kritischer t-W	2,201	
SS compared to HH		
Zweistichproben t-Test unter der Annahme		
	Variable 1	*Variable 2*
Mittelwert	0,000	0,045
Varianz	0,000	0,009
Beobachtunge	6,000	13,000
Hypothetische	0,000	
Freiheitsgrade	12,000	
t-Statistik	-1,683	
P(T<=t) einsei	0,059	
Kritischer t-W	1,782	
P(T<=t) zweise	0,118	
Kritischer t-W	2,179	

Appendix 25: A 2.69 progeny mapping *Rpgaq5* grouped according to M6 genotype segregation. VIS/cm^2 is color shaded green= low infection and red=high infection. **B**: Average VIS/cm^2 according to the genotype group and Chi – Square Test to detect segregation distortion. **C:** Student's T Test to compare means between Susceptible VV and positive control RIL 152, test VV compared to SS, and test HH compared to VV. ($p<0.05$)

6.26. Appendix 26

A

Sample	VIS/cm^2	028 (M15)	615 (M21)
2.31.27.2	1,36	v	h
2.31.27.6	1,59	v	h
2.31.27.8	1,90	v	h
2.31.27.9	1,52	v	h
2.31.27.10	2,12	v	h
2.31.27.12	2,06	v	h
2.31.27.14	3,33	v	h
2.31.27.15	2,12	v	h
2.31.27.5	2,19	v	s
2.31.27.1	1,35	v	v
2.31.27.3	3,54	v	v
2.31.27.4	3,19	v	v
2.31.27.7	1,97	v	v
2.31.27.11	1,50	v	v
2.31.27.13	1,89	v	v
152.1	3,33		
152.2	3,33		
152.3	5,85		
152.4	3,47		
143.1	0,00		
143.2	0,00		
143.3	0,00		
143.4	0,00		
143.5	0,00		

B

	VIS/cm2	
143	0,00	
ss	2,19	
hh	2,00	
vv	2,24	
152	4,00	
Expected	v	3,75
	h	7,5
	s	3,75
Observed	v	6
	h	8
	s	1
CHI		0,18268352

C

VV compared to RIL 152

Zweistichproben t-Test unter der Annahme

	Variable 1	*Variable 2*
Mittelwert	2,24035713	3,99757489
Varianz	0,82995792	1,52798193
Beobachtunge	6	4
Hypothetische	0	
Freiheitsgrade	5	
t-Statistik	-2,43606892	
P(T<=t) einsei	0,02946916	
Kritischer t-W	2,01504837	
P(T<=t) zweise	0,05893832	
Kritischer t-W	2,57058184	

VV compared to HH

Zweistichproben t-Test unter der Annahme

	Variable 1	*Variable 2*
Mittelwert	2,0	2,2
Varianz	0,4	0,8
Beobachtunge	8,0	6,0
Hypothetische	0,0	
Freiheitsgrade	8,0	
t-Statistik	-0,6	
P(T<=t) einsei	0,3	
Kritischer t-W	1,9	
P(T<=t) zweise	0,590	
Kritischer t-W	2,3	

Appendix 26: A 2.31.27 progeny mapping *Rpgaq5* grouped according to M6 genotype segregation. VIS/cm^2 is color shaded green= low infection and red=high infection. **B**: Average VIS/cm^2 according to the genotype group and Chi –Square Test to detect segregation distortion. **C:** Student's T Test to compare means between Susceptible VV and positive control RIL 152, and test HH compared to VV. ($p<0.05$)

6.27. Appendix 27

A

Sample	VIS/cm^2	028 (M15)	615 (M21)
2.40.30.1	2,96	v	h
2.40.30.7	2,50	v	h
2.40.30.8	1,67	v	h
2.40.30.10	2,86	v	h
2.40.30.12	2,99	v	h
2.40.30.15	4,59	v	h
2.40.30.6	1,67	v	s
2.40.30.11	4,42	v	s
2.40.30.14	3,00	v	s
2.40.30.2	N/A	v	v
2.40.30.3	1,31	v	v
2.40.30.4	2,50	v	v
2.40.30.5	5,97	v	v
2.40.30.9	2,08	v	v
2.40.30.13	2,02	v	v
2.40.30.16	2,14	v	v
152.1	5,71		
152.2	3,95		
152.3	6,43		
152.4	8,00		
143.1	0,14		
143.2	0,00		
143.3	0,00		
143.4	0,00		
143.5	0,00		

B

	VIS/cm2	VIS	
143	0,03		
ss	3,03		
hh	2,93		
vv	2,84		
152	6,02		
Expected	v	4	
	h	8	
	s	4	8,3
			29,6
Observed	v	7	23,8
	h	6	
	s	3	
CHI		0,22	

C

VV compared to RIL 152

Zweistichproben t-Test unter der Annahme

	Variable 1	*Variable 2*
Mittelwert	2,670	6,022
Varianz	2,770	2,828
Beobachtunge	6,000	4,000
Hypothetische	0,000	
Freiheitsgrade	7,000	
t-Statistik	-3,101	
P(T<=t) einsei	0,009	
Kritischer t-W	1,895	
P(T<=t) zweise	0,017	
Kritischer t-W	2,365	

SS compared to VV

Zweistichproben t-Test unter der Annahme

	Variable 1	*Variable 2*
Mittelwert	3,0	2,7
Varianz	1,9	2,8
Beobachtunge	3,0	6,0
Hypothetische	0,0	
Freiheitsgrade	5,0	
t-Statistik	0,3	
P(T<=t) einsei	0,4	
Kritischer t-W	2,0	
P(T<=t) zweise	0,746	
Kritischer t-W	2,6	

SS compared to HH

Zweistichproben t-Test unter der Annahme

	Variable 1	*Variable 2*
Mittelwert	3,0	2,9
Varianz	1,9	0,9
Beobachtunge	3,0	6,0
Hypothetische	0,0	
Freiheitsgrade	3,0	
t-Statistik	0,1	
P(T<=t) einsei	0,5	
Kritischer t-W	2,4	
P(T<=t) zweise	0,917	
Kritischer t-W	3,2	

Appendix 27: A 2.40.30 progeny mapping *Rpgaq5* grouped according to M6 genotype segregation. VIS/cm^2 is color shaded green= low infection and red=high infection. **B**: Average VIS/cm^2 according to the genotype group and Chi – Square Test to detect segregation distortion. **C:** Student's T Test to compare means between Susceptible VV and positive control RIL 152, test VV compared to SS, and test HH compared to VV. ($p<0.05$)

6.28. Appendix 28

A

Sample	VIS/cm^2	028 (M15)	615 (M21)
2.4.43.1	4,00	v	v
2.4.43.2	3,59	v	v
2.4.43.3	2,50	v	v
2.4.43.4	2,50	v	v
2.4.43.5	2,67	v	v
2.4.43.6	4,06	v	v
2.4.43.7	3,33	v	v
2.4.43.8	2,34	v	v
2.4.43.9	1,67	v	v
2.4.43.10	2,61	v	v
2.4.43.11	2,34	v	v
2.4.43.12	1,12	v	v
2.4.43.13	1,40	v	v
2.4.43.14	1,97	v	v
2.4.43.15	1,06	v	v
152.1	4,37		
152.2	7,82		
152.3	4,97		
152.4	8,43		
143.1	0,32		
143.2	0,00		
143.3	0,15		
143.4	0,00		
	VIS/cm2		
143	0,12		
vv	2,48		
152	6,40		

Zweistichproben t-Test unter der Annahme

VV compared to RIL 152		
	Variable 1	*Variable 2*
Mittelwert	2,476	6,397
Varianz	0,915	4,101
Beobachtunge	15,000	4,000
Hypothetische	0,000	
Freiheitsgrade	3,000	
t-Statistik	-3,762	
P(T<=t) einsei	0,016	
Kritischer t-W	2,353	
P(T<=t) zweise	0,033	
Kritischer t-W	3,182	

B

Appendix 28: A 2.40.30 progeny mapping *Rpgaq5* grouped according to M6 genotype segregation. VIS/cm^2 is color shaded green= low infection and red=high infection. **B**: Average VIS/cm^2 according to the genotype group

6.29. Appendix 29

Zweistichproben t-Test unter der Annahme		
2.14 compared to 2.54		
	Variable 1	*Variable 2*
Mittelwert	3,131	13,561
Varianz	4,340	18,591
Beobachtunge	28,000	13,000
Hypothetische	0,000	
Freiheitsgrade	15,000	
t-Statistik	-8,284	
P(T<=t) einsei	0,000	
Kritischer t-W	1,753	
P(T<=t) zweise	0,000	
Kritischer t-W	2,131	

6.30. Appendix 30

Sample	J1	J2	J3
2.14.14	s	h	h
2.54.59	s	h	h
2.27.8	s	h	h
2.60.36	v	h	h
2.60.59	v	h	h
2.27.37	v	h	h
2.14.24	v	v	h
2.58.2	h	v	v
2.60.47	h	v	v
2.58.51	h	v	v

Appendix 30: heterozygous individuals with recombinantion in 1cM interval identified to harbor *Rpgaq1*.

6.31. Appendix 31

Sample	M15	M17	J6
2.31.27	v	v	h
2.11.2	v	v	h
2.1.18	h	v	v
2.40.14	h	v	v
2.15.4	h	Unknown	v
2.11.7	h	h	v
2.19.2	h	h	v
2.5.27	h	h	v
2.40.1	h	h	v
2.5.33	h	h	s
2.31.22	h	h	s
2.11.19	h	s	s
2.19.30	s	s	h
2.1.15	s	s	h
2.40.5	s	s	h
2.4.47	s	s	h
2.40.1.1	s	Unknown	v
2.40.1.3	s	Unknown	v
2.40.1.4	s	Unknown	v
2.11.7.4	s	Unknown	v
2.11.7.12	s	Unknown	v

Appendix 31: heterozygous and homozygous individuals with a recombinantion in the 1cM interval identified to harbor *Rpgaq5*.

6.32. Appendix 32

F_2 Sample	VIS	Genotype
2-8	0	h
2-43	0	h
2-37	0	h
2-3	0	h
2-29	0	h
2-25	0	h
2-10	0	h
2-68	1	h
2-67	1	h
2-38	1	**h**
2-34	1	h
2-12	1	**h**
2-9	2	h
2-59	2	h
2-40	2	h
2-6	3	h
2-15	3	h
2-41	4	h
2-31	4	h
2-4	5	h
2-27	5	h
2-52	6	h
2-28	7	h
2-60	8	h
2-53	9	h
2-70	10	h
2-20	12	h
2-69	14	h
2-49	15	h
2-18	15	h
2-14	18	h
2-58	21	h
2-17	21	h
2-56	23	h
2-54	24	h
2-21	24	h
2-26	25	h
2-45	26	h
2-16	40	h
2-2	41	h

Appendix 32: F_2 *Rpgaq1* mapping population heterozygous genotype group ranked according to VIS.

A

Sample	VIS/cm^2	557 (J6)	615 (M21)
2.4.48.2	0,00	s	v
2.4.48.6	0,00	s	v
2.4.48.13	0,00	s	v
2.4.48.19	0,00	s	v
2.4.48.22	0,00	s	v
2.4.48.33	0,00	s	v
2.4.48.31	0,11	s	v
2.4.48.12	0,13	s	v
2.4.48.10	0,12	h	v
2.4.48.14	0,00	h	v
2.4.48.16	0,00	h	v
2.4.48.18	0,00	h	v
2.4.48.20	0,00	h	v
2.4.48.21	0,00	h	v
2.4.48.24	0,00	h	v
2.4.48.25	0,00	h	v
2.4.48.26	0,00	h	v
2.4.48.28	0,00	h	v
2.4.48.30	0,19	h	v
2.4.48.4	0,00	h	v
2.4.48.9	0,14	h	v
2.4.48.11	0,13	v	v
2.4.48.17	0,39	v	v
2.4.48.5	0,43	v	v
2.4.48.27	0,52	v	v
2.4.48.7	0,71	v	v
2.4.48.1	0,82	v	v
2.4.48.15	0,86	v	v
2.4.48.29	1,04	v	v
2.4.48.23	1,12	v	v
2.4.48.3	1,46	v	v
2.4.48.8	2,21	v	v
2.4.48.32	2,98	v	v
143.1	0,00		
143.2	0,00		
143.3	0,11		
143.4	0,00		
143.5	0,12		
152.1	9,71		
152.2	8,00		
152.3	4,11		
152.4	5,33		
152.5	1,67		

B

	Avg. VIS	Avg VIS/cm	VIS/cm
143	0,4	0,0	0,0
s	0,3	0,0	0,0
h	0,3	0,0	0,0
v	8,4	0,8	1,1
152	27,8	3,7	5,8
Expected	v	8,25	
	h	16,5	
	s	8,25	
Observed	v	12	
	h	13	
	s	8	
CHI		0,29	

C

VV compared to RIL 152

Zweistichproben t-Test unter der Annahme u

	Variable 1	*Variable 2*
Mittelwert	1,055	5,764
Varianz	0,672	10,081
Beobachtunge	12,000	5,000
Hypothetische	0,000	
Freiheitsgrade	4,000	
t-Statistik	-3,271	
P(T<=t) einseit	0,015	
Kritischer t-W	2,132	
P(T<=t) zweise	0,031	
Kritischer t-W	2,776	

SS compared to VV

Zweistichproben t-Test unter der Annahme u

	Variable 1	*Variable 2*
Mittelwert	0,0	1,1
Varianz	0,0	0,7
Beobachtunge	8,0	12,0
Hypothetische	0,0	
Freiheitsgrade	11,0	
t-Statistik	-4,3	
P(T<=t) einseit	0,0	
Kritischer t-W	1,8	
P(T<=t) zweise	0,001	
Kritischer t-W	2,2	

SS compared to HH

Zweistichproben t-Test unter der Annahme u

	Variable 1	*Variable 2*
Mittelwert	0,0	0,0
Varianz	0,0	0,0
Beobachtunge	8,0	13,0
Hypothetische	0,0	
Freiheitsgrade	17,0	
t-Statistik	-0,1	
P(T<=t) einseit	0,4	
Kritischer t-W	1,7	
P(T<=t) zweise	0,886	
Kritischer t-W	2,1	

Appendix 33: A 2.4.48 progeny mapping *Rpgaq5* grouped according to M6 genotype segregation. VIS/cm^2 is color shaded green= low infection and red=high infection. **B**: Average VIS/cm^2 according to the genotype group and Chi –Square Test to detect segregation distortion. **C:** Student's T Test to compare means between Susceptible VV and positive control RIL 152, test VV compared to SS, and test HH compared to VV. ($p<0.05$)